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(54) Title: PROTEIN- AND PEPTIDE-METAL ION PHARMACEUTICAL APPLICATIONS

(57) Abstract

Peptides containing a biological-function domain and a medically useful metal ion-binding domain and proteins containing monosulfide or disulfide bonds are labeled with medically useful metal ions for use in diagnosis and treatment of a variety of pathologic conditions. The peptides have the amino acid sequence (R_1)-$[Y_1]_n$-(R_2), (R_1)-$[Y_1$-(R_2)-$Y_1]_n$-(R_3), and (R_1)-$[Y_1$-(R_2)-$Y_2]_n$-(R_3), with the biological-function domain optionally containing the sequence IKVAV and YIGSR. The medically useful metal ion-binding domain is $[Y_1]_n$, $[Y_1$-(R_2)-$Y_1]_n$ or $[Y_1$-(R_2)-$Y_2]_n$. Sn(II) agents are used to label the proteins with medically useful metal ions.

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PROTEIN- AND PEPTIDE-METAL ION PHARMACEUTICAL APPLICATIONS

BACKGROUND OF THE INVENTION

Field of the Invention (Technical Field):

This invention relates to protein-based and peptide-based metal ion-labeled compositions for use as pharmaceuticals, and methods of labeling peptides, proteins and other similar substances with radiometals, paramagnetic metals and other medically useful metal ions, and further providing for use of medically useful metal ion-labeled peptides for detection of thrombus, cancer, infection, inflammation and various lung diseases, pathologies and abnormalities.

Description of the Related Art (Background Art):

The use of proteins, particularly antibodies, as biologically active targeting agents for medically useful metal ions has been explored. These products can be administered to the human body to visualize or monitor functioning of various parts of the body or to determine the presence and location of particular antigens, antibodies, hormones or the like; and can be used in the treatment of various disease states. Antibodies and antibody fragments have been labeled with a number of radionuclides for use in clinical diagnosis. Radionuclides commonly used include ^{131}I, ^{125}I, ^{123}I, ^{99m}Tc, ^{67}Ga, and ^{111}In for diagnostic imaging; and radionuclides such as ^{90}Y, ^{188}Re, and ^{186}Re, and to a lesser extent, ^{199}Au, ^{131}I and ^{67}Cu for targeted therapy, primarily in the treatment of cancer. There are also useful metals for magnetic resonance imaging, including gadolinium, manganese, copper, iron, gold and europium, which are not radioisotopes. So far, limited work have been done with labeling with positron-emitting radiometals, although some types of proteins, such as transferrin and human serum albumin, have been labeled with ^{68}Ga.

Antibody Labeling

Two primary methods have been employed to label antibodies with radiometals, with particular emphasis having been placed on radiolabeling with ^{99m}Tc. In one method, bifunctional chelates are conjugated to the antibody, and the bifunctional chelate is then radiolabeled. A variety of bifunctional chelates have been employed; most involve metal ion binding to thiolate groups, and may also involve metal ion binding to amide, amine or carboxylate groups. Representative bifunctional chelates include ethylenediamine tetraacetic acid (EDTA), diethylenetetramine-pentaacedic acid (DTPA), chelates of diamide-dimercaptides (N_2S_2), and variations on the

foregoing, such as chelating compounds incorporating N_2S_3, N_2S_4 or N_2S_5 metal binding sites, and metallothionine. The alternative method of radiolabeling antibodies involves reduction of disulfide bonds in the protein, with subsequent binding of the metal ion to thiolate groups. A variety of reducing agents have been employed, including stannous salts, dithiothreitol and 2-mercaptoethanol.

Antibodies and antibody fragments have been labeled with a number of radionuclides for use in clinical diagnosis. These radionuclides include ^{131}I, ^{125}I, ^{123}I, ^{99m}Tc, ^{67}Ga, and ^{111}In. So far, only ^{99m}Tc and ^{111}In-labeled antibody preparations are widely used in clinical settings. For diagnostic imaging, both isotopes should be ideal; however, clinical limitations, including affinities for liver and kidneys that limit detection of abdominal diseases, have prompted searches for other imaging radionuclides.

Antibodies have also been labeled with a variety of radionuclides for potential use in targeted immunotherapy (Pietersz GA, Kannellos J, Smyth MJ, *et al*. The use of monoclonal antibody conjugates for the diagnosis and treatment of cancer. *Immunol Cell Biol* 65:111-125, 1987). These radionuclides include ^{90}Y, ^{188}Re, and ^{186}Re, and to a lesser extent ^{199}Au and ^{67}Cu. ^{131}I has also been used. With the exception of ^{131}I, all the methods currently used to conjugate these radiometals to antibodies involve the use of chelating groups chemically attached to the antibody. ^{67}Cu is one radionuclide that has been specifically recommended for use as a therapeutic radionuclide when bound to antibodies (DeNardo GL, Raventos A, Hines HH, *et al*. Requirements for a treatment planning system for radioimmunotherapy. *Int J Radiol Oncology Biol Phys* 11:335-348, 1985). ^{199}Au-conjugated monoclonal antibodies have also been suggested for potential use as cancer therapeutic agents.

^{67}Cu has been attached to monoclonal antibodies through chelates, e.g., a macrocycle chelate (6-para-nitrobenzyl-1,4,8,11-tetraazacyclotetradecane-N,N',N",N'") (Deshpande SV, DeNardo SJ, Meares CF, *et al*. Copper-67-labeled monoclonal antibody Lym-1, A potential radiopharmaceutical for cancer therapy: labeling and biodistribution in RAJI tumored mice. *J Nucl Med 29*:217-225, 1988), and porphyrins (Roberts JC, Figard SD, Mercer-Smith JA, *et al*. Preparation and characterization of copper-67 porphyrin-antibody conjugates. *J Immunol Meth* 105:153-164, 1987). The macrocycle chelate, but not the porphyrin conjugate, was evaluated in an animal model system. Both ^{64}Cu and ^{67}Cu, have been conjugated by the porphyrin method to antibodies and autoantigenic peptides (Roberts JC, Newmyer SL, Mercer-Smith JA, Schrerer and Lavallee DK. Labelling antibodies with copper radionuclides using N-4-nitrobenzyl-5-(4-carboxyphenyl)-10,15,20-tris(4-sulfophenyl) porphine. *Appl Radiat*

Isot 40:775-781, 1989). Biodistribution studies of radiocopper-labeled antibodies have shown that blood clearance is rapid and uptake to the bone is low (Mercer-Smith JA, Cole DA, Roberts JC, *et al*. The biodistribution of radiocopper-labeled compounds. In: C Kies (ed), *Copper Bioavailability and Metabolism,* pp 103-121, 1990).

Antibodies have been labeled with ^{199}Au, in the form of gold clusters (Hainseld JF, Foley CJ, Srivastava SC, *et al*. Radioactive gold cluster immunoconjugates: Potential agents for cancer therapy. *Nucl Med Biol* 17:287-294, 1990), and with ^{199}Au and ^{195}Au, as complex ions in citrate buffered saline (Anderson P, Vaugan ATM, and Varley NR. Antibodies labeled with ^{195}Au: Potential use of ^{199}Au for radioimmunotherapy. *Nucl Med Biol 15*:293-297, 1988).

Antibodies and other proteins have been directly labeled. Although several direct methods have been reported, the first direct method capable of providing a sufficiently strong bond between the protein and technetium-99m for *in vivo* applications was the direct or pretinning method described in U.S. Pat. No. 4,424,200, entitled *Method for Radiolabeling Proteins with Technetium-99m,* to Crockford, D.R., and Rhodes, B.A. In this method, a single reduction compound, consisting of stannous [Sn(II)] chloride and other salts which serves both to reduce the protein, thereby exposing the disulfide bonds, and to reduce the sodium pertechnetate, is used. With this method, many proteins can be successfully radiolabeled with ^{99m}Tc. Several investigators have reported on the use of this method (Rhodes, B.A., et al, "Technetium-99m labeling of murine monoclonal antibody fragments," *J Nucl Med* 27:685-693, 1986; Som, P., et al, "Radioimmunoimaging of experimental thrombi in dogs using technetium-99m-labeled monoclonal antibody fragments reactive with human platelets," *J Nucl Med* 27:1315-1320, 1987).

Equivalent methods for direct labeling have been reported (Schwarz, A., and Steinstruaber, A., "A novel approach to Tc-99m-labeled monoclonal antibodies," *J Nucl Med* 28:721, 1987; Pak, K.Y., et al, "A rapid and efficient method for labeling IgG antibodies with Tc-99m and comparison to Tc-99m Fab'". *J Nucl Med* 30:793, 1989; Granowska, M., et al, "A Tc-99m-labeled monoclonal antibody, PR1A3, for radioimmunoscintigraphy," *J Nucl Med* 30:748, 1989). In the equivalent methods disulfide reducing agents other than stannous salts were used. Pak et al used dithiothreitol to reduce the disulfide bonds of the antibody; Swartz and Steinsbruaber, and Granowska et al used 2-mercaptoethanol. Also some of these investigators (Swartz and Steinsbruaber, and Granowska et al) reduced the Tc-99m prior to adding it to the reduced antibody, which adds steps to the original procedure.

Reno, J.W., et al, U.S. Pat. No. 4,877,868, *Radionuclide Antibody Coupling*, uses dithiothreitol (DTT) to reduce the disulfide groups of the protein, then protect the reactive sulfides with Zn (II) or other sulfhydryl group derivatizing reagents. Tartrate salts are used to complex and transfer the reduced radionuclide. This method uses potentially toxic chemicals, such as dithiothreitol, to reduce the antibody. It also requires multiple steps to radiolabel the protein.

Thakur, M.L., U.S. Pat. No. 5,011,676, *Method to Directly Radiolabel Antibodies for Diagnostic Imaging and Therapy*, used sodium ascorbate to reduce the disulfide groups of antibodies. However, this method cannot be adapted to single-step, direct labeling; it is required to reduce the radionuclide prior to adding the radionuclide to the sodium ascorbate reduced protein. In a preferred embodiment of the Thakur method, a separate vial is utilized, in which sodium dithionite is used to reduce the radionuclide, producing dithionite reduced radionuclide.

There are useful metals for magnetic resonance imaging, including gadolinium, maganese, copper, iron, gold and europium, which are not radioisotopes. Examples also include ions of a lanthinide element of atomic numbers 57-70 or ions of transition metals of atomic numbers 21-29 and 42-44. Examples of metals which would be expected to be of potential utility in magnetic resonance imaging with proteins labeled by the methods described in the present invention include copper, iron and gold, as well as colloidal preparations of iron or gold.

So far, antibodies do not appear to have been labeled with positron-emitting radiometals, although other types of proteins (transferrin and human serum albumin) have been labeled with ^{68}Ga (Green MA, and Welch MJ. Gallium radiopharmaceutical chemistry. *Nucl Med Biol* 16:435-448, 1989). The short half-life associated with ^{68}Ga, i.e., 68 minutes, suggests that it often may not be a suitable label for targeting antibodies, which tend to have prolonged biological half-lives.

Peptides as Radiopharmaceuticals

The use of biologically active peptides, which are peptides which bind to specific cell surface receptors, has received some consideration as radiopharmaceuticals. Canadian Patent Application 2,016,235, *Labeled Chemotactic Peptides to Image Focal Sites of Infection or Inflammation*, teaches a method of detecting a site of infection or inflammation, and a method for treating such infection or inflammation, by administration of a labeled or therapeutically-conjugated chemotactic peptide. In this application, the chemotactic

peptides are chemically conjugated to DTPA and subsequently labeled with ^{111}In. The utility of DTPA chelates covalently coupled to polypeptides and similar substances is well known in the art. Hnatowich, DJ, U.S. Pat. Nos. 4,479,930 and 4,668,503. Other bifunctional chelates for radiolabeling peptides, polypeptides and proteins are well known in the art. Other biologically active peptides described include that disclosed by Olexa SA, Knight LC and Budzynski AZ, U.S. Pat. No. 4,427,646, *Use of Radiolabeled Peptide Derived From Crosslinked Fibrin to Locate Thrombi In Vivo*, in which iodination is discussed as a means of radiolabeling. In Morgan CA Jr and Anderson DC, U.S. Pat. No. 4,986,979, *Imaging Tissue Sites of Inflammation*, use of chelates and direct iodination is disclosed. In Tolman GL, U.S. Pat. No. 4,732,864, *Trace-Labeled Conjugates of Metallothionein and Target-Seeking Biologically Active Molecules*, the use of metallothionein or metallothionein fragments conjugated to a biologically active molecule, including peptides, is disclosed. The previous methods all employ some conjugation means with a bifunctional chelator in order to effectuate labeling with a radionuclide or other medically useful metal ion, such as a paramagnetic contrast agent. The only exception involves radioiodination; the iodine labeling of proteins or peptides containing tyrosine or histidine residues is well known, for example, by the chloramine-T, iodine monochloride, Iodogen or lactoperoxidase methods.

Other biologically active peptides include analogs of formyl peptide chemoattractants which bind to neutrophils. These peptides are based on the sequence N-formyl-Met-Leu-Phe. The "C" terminal end can be modified to include additional sequences constituting a metal ion binding domain. The clinical and diagnostic imaging potential of formylated chemotactic peptides has recently been demonstrated by Fischman et al. (Fischman AJ, Pike MC, Kroon D, Fucello AJ, Rexinger D, tenKate C, Wilkinson R, Rubin RH and Strauss HW: Imaging focal sites of bacterial infection in rats with indium-111-labeled chemotactic peptide analogs. *J Nucl Med* 32:483-491, 1991) using chemotactic peptides chemically conjugated to DTPA and subsequently labeled with ^{111}In. Chemotactic peptides have also been radioiodinated by synthesizing formylated peptides containing tyrosine amino acids. These peptides have been used *in vitro* and have the same biological function as unlabeled formylated peptides (Janeczek AH, Marasco WA, Van Alten PJ and Walter RB: Autoradiographic analysis of formylpeptide chemoattractant binding, uptake and intracellular processing by neutrophils. *J Cell Sci* 94:155-168, 1989).

Peptide analogues of somatostatin have been used after radiolabeling for diagnostic imaging. Somatostatin is a hormone produced by the hypothalamus which normally inhibits the release of

pituitary growth hormone. A number of peptide analogues have been developed which have pharmacological actions that mimic the naturally-occurring hormone. Octreotide acetate, one of the somatostatin analogues, has a disulfide bond in it. In normal subjects somatostatin and its analogues have the ability to suppress secretion of serotonin and the gastroenteropancreatic peptides, and growth hormone. A number of tumor types have been found to express somatostatin receptors, with ^{125}I-labeled somatostatin analogues used to image small-cell lung cancer (Kwekkeboom DJ, Krenning EP, Bakker WH et al: Radioiodinated somatostatin analog scintigraphy in small-cell lung cancer. *J Nucl Med* 32:1845-1848, 1991).

The potential role of amino acid sequences found in peptides and proteins in binding transition metals has been recognized. In Vallee BL and Auld DS: Zinc coordination, function, and structure of zinc enzymes and other proteins, *Biochemistry* 29:5648-5659, 1990, the general characteristics of non-metallothionein proteins which contain zinc binding sites are described. Arnold FH and Haymore BL describe histidine-containing amino acid sequences used for protein purification by metal-chelate chromatography (Engineered metal-binding proteins: purification to protein folding, *Science* 252:1796-1797, 1991). Iverson et al. describe a means of genetic manipulation of antibodies to contain metal binding sites in the immunological binding region with the goal of producing catalytic antibodies (Iverson BL, Iverson SA, Roberts VA, Getzoff ED, Tainer JA, Benkovic SJ and Lerner RA: Metalloantibodies, *Science* 249:659-662, 1990). The use of histidine-containing amino acid sequences which bind Ru to form exchange-inert metal complexes to form highly stable α-helical metallopeptides was described in Ghardiri MR and Fernholz AK: Peptide architecture. Design of stable α-helical metallopeptides via a novel exchange-inert Ru^{III} complex, *J Am Chem Soc* 112:9633-9635, 1990. The role of isolated amino acid ligands to bind ^{99}Tc and ^{99m}Tc has long been recognized; Seifert et al. describes the capability of nitrogen donor atoms to stabilize reduced technetium species using free lysine, ornithine and histidine (Seifert S, Munze R and Johannsen B: Technetium-99 and 99m chelates with N-donor ligands: a new class of potentially cationic radiopharmaceuticals, in *Technetium in Chemistry and Nuclear Medicine* Deutsch E, Nicolini M and Wagner HN Jr, eds., Cortina International, Verona, 1983, pp 19-23.

Lung Imaging

Pulmonary radionuclide imaging techniques currently in general practice involve the use of a) macroaggregated albumin or albumin microspheres (MAA), b) radioaerosols (^{99m}Tc-DTPA), c) radioactive gases, and d) gallium citrate. *See*, generally, Anger, K: Radionuclide Studies of the Lung, In: Sperber, M. (editor), Radiologic Diagnosis of Chest Disease, Springer-Verlag, New York,

1990, pp 140-153; Miller RF and O'Doherty MJ: Pulmonary nuclear medicine. *Eur J Nucl Med* 19(1992) 355-368. MAA is a radioactive particle which is sequestered by capillary blockade. After administration of radioactive particles greater than 10 μm in diameter into a peripheral vein, the pulmonary capillaries and precapillary arterioles act like a sieve, with the ^{99m}Tc-MAA particles temporarily blocked so that the tracer is trapped in its first passage through the lung. The radioactivity distribution reveals relative pulmonary perfusion. When blood flow has been interrupted or significantly changed in a portion of the lung larger than 2 cm, a defect appears as a photon-deficient image. Most of the MAA particles (90%) have a diameter ranging from 10-40 μm. MAA particles degrade into smaller particles leaving the lung vasculature with a biologic half-time of 2-9 hours, and are cleared by phagocytosis in the reticuloendothelial system.

Many pulmonary diseases produce an altered pulmonary blood flow in the affected areas. Radiolabeled MAA allows detection of areas of altered blood flow, but provides no information related to any specific biochemical or metabolic event. Perfusion lung scintigraphy is highly sensitive, but not specific. Moreover, perfusion defects (13%) have been found in non-smoking volunteers without pulmonary disease, and loss of normal apex-to-base gradients (9%) have also been observed (Webber MM, Renick LH, Fouad BI, and Victery WK: Variants of the normal lung scan: Correlation with pulmonary function tests. *J Nucl Med* 13(1972) 476; Tetalman MR, Hoffer PB, Heck LL, et al: Perfusion scans in normal volunteers. *Radiology* 106(1973) 593-594). The lack of specificity can lead to mis-diagnosis, the need for additional test procedures, and delays in implementing therapy. Thus, there is a need for a diagnostic radiopharmaceutical which can overcome some or all of these problems.

The lung is an organ which can undergo extensive degradation and remodeling of the extracellular matrix as a result of disease. Emphysema, fibrosis, cancer and other chronic obstructive lung diseases all can lead to both microscopic and macroscopic alterations in air space, and related changes in the lung extracellular matrix and basement membrane. A radiopharmaceutical which can detect specific alterations in extracellular molecules or their receptors can be used as a specific probe of the biochemical and metabolic status of the lung in disease processes.

Laminin is a basement membrane glycoprotein (M_r = 900,000) which has various biological activities including promoting cell attachment, growth, and differentiation. A typical laminin molecule consists of three polypeptide chains -- A (440 kd), B1 (200 kd), and B2 (220 kd) -- that are linked by disulfide bonds to form an

asymmetric cross-structure. Multiple, distinct adhesive sequences in laminin appear to mediate specific biological functions, and bind to distinct cell surface receptors (Hynes RO: Integrins: versatility, modulation, and signaling in cell adhesion, *Cell* 69(1992) 11-25; Yamada KM: Adhesive recognition sequences, *J Biol Chem* 266(1992) 2809-2812).

One adhesive sequence from the laminin A-chain is Ile-Lys-Val-Ala-Val (IKVAV), and this peptide as well as longer laminin peptide sequences containing IKVAV have been reported to increase in vitro adhesiveness of a number of cell lines including mast cells (Thompson HL, Burbelo PD, Yamada Y, Kleinman HK, and Metcalfe DD: Identification of an amino acid sequence in the laminin A chain mediating mast cell attachment and spreading, *Immunology* 72(1991) 144-149; Thompson HL, Burbelo PD, Yamada Y, Kleinman HK, and Metcalfe DD: Mast cells chemotax to laminin with enhancement after IgE-mediate activation, *J Immunol* 143(1989) 4188-4192), cerebral cells (Tashiro K-I, Sephel GC, Weeks B, Sasaki M, Martin GR, Kleinman HK, and Yamada Y: A synthetic peptide containing the IKVAV sequence from the A chain of laminin mediates cell attachment, migration, and neurite outgrowth, *J Biol Chem* 27(1989) 16174-16182; Kleinman HK, Weeks BS, Cannon FB, Sweeney TM, Sephel GC, Clement B, Zain M, Olson MOJ, Jucker M, Burrous BA: Identification of a 100-kDa nonintegrin cell surface laminin-binding protein which recognizes an A chain neurite-promoting peptide. *Arch Biochem Biophys* 290(1991) 320-325; Skubitz APN, Letourneau PC, Wayner E, and Furcht LT: Synthetic peptides from the carboxyl-terminal globular domain of the A chain of laminin: their ability to promote cell adhesion and neurite outgrowth, and interact with heparin and the B1 integrin subunit, *J Cell Biol* 115(1991) 1137-1148; Sephel GC, Tashiro K-I, Sasaki M, Greatorex D, Martin GR, Yamada Y, and Kleinman HK: Laminin A chain synthetic peptide which supports neurite outgrowth, *Biochem Biophys Res Comm* 162(1989) 821-829), normal mesenchymal cells (Kleinman et al., *supra*, 1991), tumor cells (Kleinman et al., *supra*, 1991), and hepatocytes (Clement B, Segui-Real B, Savagner P, Kleinman HK, and Yamada Y: Hepatocyte attachment to laminin is mediated through multiple receptors, *J Cell Biol* 110(1990) 185-192). One such longer peptide, Cys-Ser-Arg-Ala-Arg-Lys-Gln-Ala-Ala-Ser-Ile-Lys-Val-Ala-Val-Ser-Ala-Asp-Arg (also referred to as PA22-2), increased in vivo lung colonization by melanoma cells (Kanemoto T, Reich R, Royce L, Greatorex D, Adler SH, Shiraishi N, Martin GR, Yamada Y, and Kleinman HK: Identification of an amino acid sequence from the laminin A chain that stimulates metastasis and collagenase IV production, *Proc Nat Acad Sci (USA)* 87(1990) 2279-2283). The conformational status, but not specific chirality, of the IKVAV domain is a contributing factor in biological activity (Nomizu M, Utani A, Shiraishi N, Kibbey MC, Yamada Y, and Roller PR: The all-D-configuration segment containing

the IKVAV sequence of laminin A chain has similar activities to the all-L-peptide *in vitro* and *in vivo*, *J Biol Chem* 267(1992) 14118-14121).

Thrombus Imaging

Under homeostatic conditions, platelets circulate as disc shaped cells that do not interact with other circulating blood cells or vascular endothelium (Buchanan MR: Mechanisms of pathogenesis of arterial thrombosis: potential sites of inhibition by therapeutic compounds, *Sem Thrombosis and Hemostasis* 14(1988) 33-40). The release of adhesive and coagulant agents associated with platelet activation is held in check by high intraplatelet, and possibly vascular endothelium, levels of cAMP.

Upon injury, platelets rapidly attach a) to dysfunctional or detached endothelial cells and b) to the underlying basement membrane and tissues. Differences in platelet response, correlating to the degree of injury, are due in part to differences in the vessel wall composition of the molecules to which the platelets adhere. For example, type I and III collagens, which are typically associated with smooth muscle cells, promote platelet adhesion, aggregation, and release. In contrast, types IV and V collagens, typically associated with the endothelium, facilitate platelet adhesion but do not generally cause platelet activation.

Platelet-mediated thrombosis is a major pathogenetic mechanism in thrombogenesis and reocclusion after successful thrombolytic therapy, and consequently platelets are frequently used as vehicles for localization of thrombi. Additionally, suppression of platelet aggregation is a frequent target for prevention of blood vessel occlusion or reocclusion. There are a number of clinical conditions in which there are platelet accumulations; these include venous thrombosis, arterial thrombosis, left ventricular thrombosis, pulmonary embolism, inflammatory response secondary to myocardial infarction, endocarditis, bypass graft occlusion, aneurysms, prosthetic arterial graft platelet accumulation or occlusion, cerebral embolism or hemorrhage, traumatic injury with hemorrhage, gastrointestinal hemorrhage, and thrombosis secondary to catheters and other implanted devices.

A variety of diagnostic modalities have been used for conditions involving platelet accumulation. These include contrast venography, impedence plethysmography, and ^{125}I-fibrinogen uptake for venous thromboembolism; ^{111}In-labeled platelets for a variety of conditions involving platelet accumulation; and, pulmonary angiography, perfusion lung scanning using ^{99m}Tc-human macroaggregated albumin, and ventilation-perfusion lung scanning with radioactive

gases or aerosols for pulmonary embolism. Each of these modalities presents serious limitations, and has less than desirable efficacy. ^{111}In-labeled platelets is the only modality which yields a reliable direct measure of platelet accumulation; however, this method suffers serious limitations, including technical difficulties in ex vivo labeling. In addition, since with ^{111}In-labeled platelets the labeling is performed ex vivo, and the platelets reinjected and allowed to accumulate before imaging, this method does not provide a measure of existing platelet accumulation. Thus, no commonly used method allows for direct detection of existing platelet accumulation within the body.

Peptides containing the adhesive sequence RGD are under active investigation as anti-thrombotic agents (Imura Y, Stassen J-M, Dunting S, Stockmans F, and Collen D: Antithrombotic properties of L-cysteine, N-(mercaptoacetyl)-D-Tyr-Arg-Gly-Asp-sulfoxide (G4120) in hamster platelet-rich femoral vein thrombosis model, *Blood* 80(1992) 1247-1253). Knight et al. (Knight LC, Radcliffe R, Kollman M, Dasika V, Wikander R, Mauer AH, Rodwell JD, and Alvarez V: Thrombus imaging with Tc-99m synthetic peptides reactive with activated platelets. *J Nucl Med* 31(1990) 757 (abstract)) have reported on the use of ^{99m}Tc-synthetic peptide-metallothionein complexes which bind to the platelet glycoprotein IIb/IIIa complex to image fresh thrombi in jugular veins. However, peptides which target the glycoprotein IIb/IIIa complex are known to adversely affect platelet aggregation, and consequently a radiopharmaceutical based on such an approach would be expected to have severe dose limitations.

In addition to peptides, radiolabeled monoclonal antibodies specific for platelet-related antigens have been studied as diagnostic radiopharmaceuticals. (Shah VO, Zamora PO, Mills SL, Mann PL, and Comp PC: In vitro studies with the platelet-reactive antibody 50H.19 and its fragments. *Thrombosis Research* 58(1990) 493-504; Som P, Oster ZH, Yamamoto K, Sacker DF, Brill AB, Zamora PO, Newell KD, and Rhodes BA: Radioimmunoimaging of experimental thrombi in dogs using Tc-99m labeled monoclonal antibody fragments reactive with human platelets. *J Nucl Med* 27(1986) 1315-1320).

Integrin-type receptors on platelets (glycoprotein Ib, the glycoprotein IIb/IIIa complex and glycoprotein IV) have been identified as the major adhesion receptors in platelets, but these glycoproteins do not appear to play a role in the interaction of platelets with the intact laminin molecule (Tandon NN, Holland EA, Kralisz U, Kleinman HK, Robey FA, and Jamieson GA: Interaction of human platelets with laminin and identification of the 67 kDa laminin receptor on platelets, *Biochem J* 274(1991) 535-542). However,

platelets do bind to laminin peptide fragments via these receptors (Sonnenberg A, Gehlsen KR, Aumailley M, and Timpl R: Isolation of $\alpha 6\beta 1$ integrins from platelets and adherent cells by affinity chromatography on mouse laminin fragment E8 and human laminin pepsin fragment, *Exp Cell Res* 197(1991) 234-244), suggesting that normally these sites in laminin are cryptic for platelets. One non-integrin platelet receptor for laminin is a 67 kDa receptor which binds to laminin-derived peptide sequences containing Tyr-Ile-Gly-Ser-Arg (YIGSR) (Tandon et al., *supra*). This platelet receptor appears to play an important role in the interaction of platelets with the intact laminin molecule. Platelet adherence to laminin via this receptor does not in itself result in platelet activation (Ill CR, Engvall E, and Ruoslahti E: Adhesion of platelets to laminin in the absence of activation. *J Cell Biol* 99(1984) 2140-2145).

Peptides containing the YIGSR peptide sequence have been proposed as anti-metastatic agents. Yamada Y, Graf JO, Iwamoto Y, Rober F, Kleinman HK, Sasaki M and Martin GR, U.S. Patent 5,092,885, *Peptides with Laminin Activity*; Schasteen CS, U.S. Patent 5,039,662, *Peptide with Anti-Metastatic Activity*. These patents involve longer sequences containing the YIGSR peptide sequence, as well as acylated YIGSR peptide sequences.

SUMMARY OF THE INVENTION (DISCLOSURE OF THE INVENTION)

In accordance with the present invention, a peptide-based pharmaceutical composition suitable for administration to a patient is provided. The composition, which may be lyophilized, includes a peptide which itself comprises a biological-function domain and a medically useful metal ion-binding domain, and further includes a metal ion labeling agent. The peptide is selected from the group consisting of

$(R_1)-[Y_1]_n-(R_2)$,

$(R_1)-[Y_1-(R_2)-Y_1]_n-(R_3)$

and $(R_1)-[Y_1-(R_2)-Y_2]_n-(R_3)$

wherein,

the medically useful metal ion-binding domain is selected from one of the group consisting of $[Y_1]_n$, $[Y_1-(R_2)-Y_1]_n$ and $[Y_1-(R_2)-Y_2]_n$ in which n is a number between 1 and about 6 and Y_1 and Y_2 are amino acids comprising a sulfur, nitrogen or oxygen which is available for binding to metal ions, or can be made available for binding to metal ions;

the biological-function domain comprises at least one of the group consisting of R_1, R_2 and R_3 and further comprises an amino acid sequence containing from 1 to about 20 amino acids; and

those portions of R_1, R_2 and R_3 not comprising the

biological-function domain each comprise an amino acid sequence containing from 0 to about 20 amino acids.

The biological-function domain may be located in any one or more of R_1, R_2 or R_3, including situations in which the biological-function domain comprises all or part of two or more of R_1, R_2 or R_3. It is not required that the biological-function domain constitute all of the amino acid sequence of any one of R_1, R_2 or R_3; that is, it is possible and contemplated that the biological-function domain will be an amino acid sequence constituting a portion of the total amino acid sequence of any one of R_1, R_2 or R_3, with the remainder of that region being an amino acid sequence which is not the biological-function domain.

The medically useful metal ion-binding domain of the peptide-based pharmaceutical composition includes amino acid sequences containing cysteine, cystine, histidine, penicillamine, deacylated methionine, lysine, arginine, aspartic acid, glutamic acid or tyrosine. Specific medically useful metal ion-binding domains include the following:

$[Cys]_n$,
$[Cys-(R_2)-Cys]_n$,
$[Cys-(R_2)-Pen]_n$,
$[His-(R_2)-Cys]_n$,
$[His-(R_2)-Pen]_n$,
$[His]_n$
and $([His-(R_2)-His]_n$
wherein,
n is a number between 1 and about 6; and
R_2 is an amino acid sequence containing from 1 to about 20 amino acids. R_2 may optionally include all or part of the biological-function domain, or the biological-function domain may be located outside of the metal ion-binding domain.

The metal ion labeling agent which is included in the peptide-based pharmaceutical composition can be a stannous ion agent, which may be present in a solution including alkali metal tartrate. The stannous ion agent can also be present in a solution including dicarboxylic acid. Representative forms of dicarboxylic acid which can be used include phthalate, tartrate and citrate. The stannous ion agent itself can include stannous tartrate, stannous glucoheptonate, stannous gluconate, stannous phosphonate, stannous chloride or stannous fluoride.

The peptide-based pharmaceutical composition can also include a medically useful metal ion, which may be radioactive, paramagnetic or superparamagnetic. The medically useful metal ion can be selected

from the group consisting of ionic forms of the elements iron, cobalt, nickel, copper, zinc, arsenic, selenium, technetium, ruthenium, palladium, silver, cadmium, indium, antimony, rhenium, osmium, iridium, platinum, gold, mercury, thallium, lead, bismuth, polonium and astatine. The medically useful metal ion can also be a radionuclide comprising an isotope selected from the group consisting of indium, gold, silver, mercury, technetium, rhenium and copper.

DETAILED DESCRIPTION OF THE PREFERRED EMBODIMENTS OF THE INVENTION (BEST MODES FOR CARRYING OUT THE INVENTION)

Using the methods of this invention, peptides and proteins containing metal ion binding sequences can be coupled directly with metal ions to provide materials useful for in vivo diagnostic and therapeutic applications. Peptides may be used alone, in combination with other peptides or may be chemically-conjugated to a host molecule. The peptides can be prepared in a format providing a labeling kit which can, in turn, be used to prepare a metal ion-peptide complex for in vivo use. The peptides of this invention contain:

a) biological-function domains, and

b) metal ion-binding domains which can complex with medically useful metal ions.

The biological-function domain of the peptide is defined in the specification and claims as a sequence of one or more amino acids which exhibit binding to a biological receptor found on cells, tissues, organs or fluids. The peptides may or may not transmit a signal to the cells, tissues or other materials associated with the biological receptor after binding. The biological-function domain also includes a sequence of one or more amino acids which exhibit binding to a biological receptor found on other peptides, enzymes, antibodies or similar proteinaceous compositions which may themselves exhibit binding to another biological receptor.

The metal ion-binding domain of the peptide is defined in the specification and claims as a sequence of one or more amino acids containing sulfur, nitrogen or oxygen which is available for binding or can be made available for binding to metal ions. Sulfur-containing amino acids include primarily cysteine (Cys), cystine (Cys-Cys) and penicillamine (Pen), although deacylated methionine (Met) may also be used. Nitrogen-containing amino acids include primarily histidine (His), but under certain conditions lysine (Lys) and arginine (Arg), which have pK_a values of 10.0 and 12.0, may also be employed. In addition, the terminal amino group of peptides may also be employed. Oxygen-containing amino acids include aspartic acid (Asp), glutamic acid (Glu) and tyrosine (Tyr), as well as the terminal carboxyl group of peptides. The amino acid sequences most

usefully employed will include one or more Cys, one or more His, or a combination of Cys and His. Pen, which is an analogue of Cys, may be directly substituted for any given Cys. Cys may be present in the peptide as a disulfide in the form of cystine. The metal ion-binding domains may occur once or multiple times in any given peptide, and may occur in any combination. The metal ion-binding domain and the biological-function domain may overlap.

The metal binding sequences as found in the peptides of this invention are stabilized by the addition of a positively-charged transition metal ion of Zn, Cu, Sn, Co, or Ni, selected to have a low order of binding strength. Through a replacement reaction, the transition metal ion replaces the H ion of the thiolate, imidazole or carboxyl group. The divalent ions of zinc and tin are thought to be particularly attractive. Some transition metals can simultaneously be used to reduce disulfide bridges and stabilize the metal binding sequences, such as Sn (II), which is particularly useful with cystine formations. In any case, the transition metals are weakly associated with the peptide.

The positively-charged transition metal ions are introduced to the peptide in an aqueous solution containing an appropriate buffer. The buffer may consist of dicarboxylic acids (tartrate, phthalate, citrate), amino acids (glycine), borate or the like. For radiolabeling in acidic conditions typically 10 mM tartrate and 40 mM phthalate, pH 5.6, are used. For radiolabeling in basic conditions typically 10 mM glycine, pH 9.0, is used. The buffer may also contain a number of excipients and/or stabilizers including NaCl, inositol, glucoheptonate, or the like.

The peptides are subsequently incubated with a medically-useful metal ion. The medically-useful metal ion is selected to have a higher order of binding than the positively charged-transition metal ion used to stabilize the metal binding sequences. A number of medically-useful metal ions can be used; radiometals include isotopes of the elements of Tc, Re, Au, Ag, Pd, As, Cu, Hg, and Ru. Radioisotopes of Tc are of significant interest, and particularly ^{99m}Tc. In the case of ^{99m}Tc, the peptides are reacted with sodium pertechnetate which has been treated with a reducing agent to generate Tc with a lower oxidation state. The product of the reaction between the metal ion and the peptide is a complex of the metal ion and the peptide. For example, the following structures could result from use of the invention, using Tc labeling of peptides containing metal-ion binding domains consisting of Cys and His groups as an example:

a) (biological-function domain)-[Cys]$_n$
|
Tc=O
|
X_n

b) (biological-function domain)-[Cys-(R)-Cys]$_n$
\ | /
Tc=O
|
X_n

c) (biological-function domain)-[His-(R)-His]$_n$
\ | /
Tc=O
|
X_n

in which R is an amino acid sequence containing from 0 to about 20 amino acids and X_n is an anion, such as a halogen like fluoride or chloride, or a solvent molecule, such as water.

The resulting Tc-peptide bond should have a sufficiently high bond strength to minimize the exchange of the radionuclide to transferrin and serum albumin. The complex should be thermodynamically stable under physiological conditions and exhibit acceptable toxicological properties.

Most stannous reductions are performed at a pH of from about 5 to about 6. With amino acid side chains in a solution at pH 5.6, the basic amino acids are positively charged, the acidic amino acids are largely negatively charged, the alcoholic amino acids are neutral, and methionine is neutral. Since reduced technetium binds more readily to neutral hydrogen donors rather than positively charged hydrogen donors, at the pH range 5 to 6 only Cys and His are optimal ^{99m}Tc binding site candidates. For both Cys and His, radiolabeling yields are dependant on pH, and are theoretically optimal at or near the pK_a.

The metal ion-peptides of this invention may be used directly for administration, or alternatively may be conjugated to a carrier or targeting molecule. The methods for conjugating peptides to carrier molecules are well known to those skilled in the art. The conjugations may involve covalent binding through carbohydrate residues, sulfhydryl residues, amine groups (including those of lysine), and carboxyl groups.

The peptides of the invention can be:

a) naturally-occurring,

b) produced by chemical synthesis,

c) produced by recombinant DNA technology,

d) produced by biochemical or enzymatic fragmentation of larger molecules,

e) produced by methods resulting from a combination of a-d, or

f) produced by any other means for producing peptides.

The peptides can also include peptide fragments, oliopeptides, polypeptides and other like structures, generally consisting of a sequence of amino acids. Representative types of peptides include those derived from laminin, fibronectin, cytokines, lymphokines, hormones, serum albumin, fibrinogen, enzymes, hormones, somatostatin, urokinase, tissue plasminogen activator, and protease inhibitors. The term "peptide" as used throughout the specification and claims is intended to include all of the foregoing.

The peptide of this invention is reacted with a medically useful metal ion. The medically useful metal ion may be radioactive and generate gamma rays, beta particles, or positrons which are converted into gamma rays upon collision with electrons. Alternatively, the medically useful metal ion may be paramagnetic. The medically useful metal ion may used in diagnostic imaging procedures including gamma scintigraphy, specific photon emission computerized tomography, or positron emission tomography. The medically useful metal ion may also be used diagnostically in magnetic resonance imaging. Medically useful metal ions may also be used therapeutically.

The type of medically useful metal ion depends on the specific medical application. Particularly useful metal ions can be found in the group consisting of elements 26-30 (Fe, Co, Ni, Cu, Zn), 33-34 (As, Se), 42-50 (Mo, Tc, Ru, Rh, Pd, Ag, Cd, In, Sn) and 75-85 (Re, Os, Ir, Pt, Au, Hg, Tl, Pb, Bi, Po, At). Isotopes of the elements Tc, Re, and Cu are particularly applicable for use in diagnostic imaging and radiotherapy. The isotope ^{99m}Tc is particularly applicable for use in diagnostic imaging. Other radionuclides with diagnostic or therapeutic applications include ^{62}Cu, ^{64}Cu, ^{67}Cu, ^{97}Ru, ^{105}Rh, ^{109}Pd, ^{186}Re, ^{188}Re, ^{198}Au, ^{199}Au, ^{203}Pb, ^{211}Pb and ^{212}Bi.

Any protein, peptide, oliopeptide, glycopeptide, glycoprotein, amino acid sequence, chelating agent or other substrate which contains one or more disulfide bonds or one or more monosulfides, including fragments of any of the foregoing or molecules formed by attaching or complexing any of the foregoing to another molecule, can also be labeled in accordance with this invention. Representative suitable substrates include human serum albumin, fibrinogen, urokinase, gamma globulin, laminin, fibronectin, cytokines, lymphokines, enzymes, enzyme inhibitors, hormones, glycoproteins, oligopeptides, peptides, both natural and synthetic, other proteins

and immunoglobulins. The term "protein" as used throughout the specification and claims is intended to include all of the foregoing substances. The protein is typically of mammalian origin, but also includes proteins of plant origin and proteins from prokaryotic cells. Methods of attaching or complexing proteins to other molecules, such as lipids and carbohydrates, including liposomes, is known to those skilled in the art.

Immunoglobulins, a type of protein, which can be labeled include antibodies and antibody fragments, of any species, and include both polyclonal and monoclonal antibodies made by any means, as well as chimeric and genetically engineered antibodies,hybrids, and fragments of all of the foregoing. This includes immunoglobulins of any class, such as IgG, IgM, IgA, IgD or IgE, of any species origin, including human beings, chimeric antibodies or hybrid antibodies with dual or multiple antigen or epitope specificities, and fragments of all of the foregoing, including $F(ab')_2$, $F(ab)_2$, Fab', Fab and other fragments, including hybrid fragments, and further includes any immunoglobulin or any natural, synthetic or genetically engineered protein that functionally acts like an antibody by binding to a specific antigen to form a complex, including single chain antibodies. The term "antibody" or "antibodies", and the phrase "monoclonal antibody component", as used throughout the specification and claims is intended to include all such antibodies and antibody fragments.

It is possible to chemically modify the protein by the introduction of monosulfides or disulfide bonds. A protein, even though it may not natively contain monosulfides or disulfide bonds, with attached or complexed disulfide bonds can be labeled in accordance with this invention. Means to attach or complex disulfide bonds, and chelating agents and substrates containing disulfide bonds, are known to those skilled in the art. Phytohemagglutinin, and the L-4 isolectin thereof, is an example of a protein that does not natively contain disulfide bonds. Disulfide bonds may be introduced into such proteins by chemical methods involving direct conjugation. Chemical means used to introduce disulfide bonds into proteins includes use of homofunctional crosslinkers, heterofunctional crosslinkers, and monofunctional protein modification agents. Representative chemicals which can be used to introduce disulfide bonds into proteins include 4-succinimidyloxy-carbonyl-alpha-methyl-alpha-(2-pyridyldithiol)-toluene; N-succinimidyl 3-(2-pyridyldithio)propionate; sulfosuccinimidyl 6-[3-(2-pyridiyldithiol) propinoamido] hexonate; dithio*bis*(succinimidylproprionate); 3,3'-dithio*bis*(sulfosuccinimidylpropionate); and sulfoscucinimidyl 2-(p-azidosalicylamido)ethyl-1,3'-dithipropionate.

The product resulting from the methods set forth herein can be used for both medical applications and veterinary applications. Typically, the product is used in humans, but may also be used in other mammals. The term "patient" is intended to denote a mammalian individual, and is so used throughout the specification and in the claims. The primary applications of the invention involve human patients, but the invention may be applied to laboratory, farm, zoo, wildlife, pet or sport animals.

The product may be used to monitor normal or abnormal metabolic events, to localize normal or abnormal tissues, to localize diseases, and to bind to blood constituents, including blood cells, such as lymphocytes, for subsequent localization of diseases, infections, and abnormal tissues. The application and medical use of the product depends on the type of peptide and the type of medically useful metal ion used.

Using the methods of this invention, peptides with a biological-function domain comprising at least the sequence IKVAV and a linked radiolabel provide materials useful for in vivo diagnostic applications, particularly for diagnostic imaging of the lungs. Preferably, the peptide comprises a biological-function domain of at least the sequence IKVAV and a metal-ion binding domain comprising metal ion binding sequences which can be coupled directly with metal ions. The peptides can be prepared in a format providing a labeling kit which can, in turn, be used to prepare a metal ion-peptide complex for in vivo use. It is also possible to provide for labeling of a peptide with the biological-function domain with a metal ion in vivo, such as through use of a peptide-avidin complex, which is injected in vivo, followed by a biotin-metal ion complex inject in vivo, resulting in formation of an in vivo peptide-avidin-biotin-metal ion complex. The IKVAV-containing peptides of this invention preferably contain:

a) biological-function domains comprising at least the sequence IKVAV, and

b) metal ion-binding domains which can complex with medically useful metal ions.

The biological-function domain of the IKVAV-containing peptide is defined in the specification and claims as a sequence of the amino acids Ile-Lys-Val-Ala-Val (IKVAV single amino acid code), and optionally amino acids in addition to IKVAV which are useful for lung imaging and treatment. The IKVAV peptide of this invention will, for the most part, include the sequence RKQAASIKVAV, and most preferably the sequence CSRARKQAASIKVAVSADR. Usually, within the indicated sequences, there may be mutations, including deletions, insertions or substitutions. It is possible that the sequence IKVAV may be

repeated one or more times, to increase localization. For the most part, substitutions will be conservative, in which amino acids having substantially the same conformation and polarity may be employed. The peptides may use L-amino acids, or one or more of the amino acids may be substituted by D-amino acids (D-stereoisomer), which may in part increase resistance to protease degradation. Particularly, one or more alanines may be substituted. In the alternative, terminal amino acids may be employed having unnatural chirality. The peptide may also include a terminal amide or a terminal acylated amino acid, particularly acetylated or alkylated, particularly methylated, amino acids. Where a cysteine provides the metal-ion binding domain at the N-terminus, the cysteine may be alkylated or unsubstituted on the mercaptan group.

It is hypothesized, without wishing to bind the inventor herein, that lung localization using the IKVAV-containing peptide is receptor-based, and due in part to pulmonary endothelial cell binding, and in some instances to tumor receptor binding. There is also evidence to suggest that IKVAV-containing peptide binds to receptors on tissue plasminogen activator, which is frequently present in relative high concentrations in tumor cells. Regardless of the exact mechanism of receptor-based lung localization, such a mechanism presents significant advantages for a ^{99m}Tc-peptide containing the IKVAV sequence over ^{99m}Tc-MAA:

a) The peptide should not itself alter pulmonary perfusion,

b) The peptide should bind to pre-capillary, capillary, and post-capillary endothelial cells and thereby, provide a more representative view of the actual physiology of the lung vasculature,

c) The use of a synthetic peptide for imaging would obviate considerations relating to viral (HIV or the like) contamination of the source material,

d) The use of a non-particulate imaging agent should minimize health risks in hyper-sensitive patient populations, such as pediatric use;

e) Differential diagnosis of certain conditions may be possible, in that chronic obstructive conditions such as emphysema will be detected as photon-deficient, and certain tumors will be detected as photon-rich.

The IKVAV-containing peptide product may be used to monitor or treat normal or abnormal tissues and metabolic events, particular chronic obstructive pulmonary disease, such as emphysema or fibrosis, in which abnormal tissues or metabolic events will generally produce a photon-deficient area, and to localize primary or metastatic cancerous tumors, and particularly cancerous tumors of the lung, in

which cancerous tumors will generally produce a photon-abundant area.

Using the methods of this invention, peptides with a biological-function domain comprising at least the sequence YIGSR and a linked radiolabel provide materials useful for in vivo diagnostic applications, particularly for diagnostic imaging of thrombosis and other conditions characterized by accumulation of platelets. Preferably, the peptide comprises a biological-function domain comprising at least the sequence YIGSR and a metal-ion binding domain comprising metal ion binding sequences which can be coupled directly with metal ions. The peptides can be prepared in a format providing a labeling kit which can, in turn, be used to prepare a metal ion-peptide complex for in vivo use. It is also possible to provide for labeling of a peptide with the biological-function domain with a metal ion in vivo, such as through use of a peptide-avidin complex, which is injected in vivo, followed by a biotin-metal ion complex inject in vivo, resulting in formation of an in vivo peptide-avidin-biotin-metal ion complex. The peptides of this invention preferably contain:

a) biological-function domains comprising at least the sequence YIGSR, and

b) metal ion-binding domains which can complex with medically useful metal ions.

The biological-function domain of the YIGSR-containing peptide is defined in the specification and claims as a sequence of the amino acids Tyr-Ile-Gly-Ser-Arg (YIGSR single amino acid code), and optionally amino acids in addition to YIGSR. The peptide of this invention thus preferably includes the sequence YIGSR, which may be repeated one or more times. Usually, within the indicated sequences, there may be mutations, including deletions, insertions or substitutions. For the most part, substitutions will be conservative, in which amino acids having substantially the same conformation and polarity may be employed. The peptides may use L-amino acids, or one or more of the amino acids may be substituted by D-amino acids (D-stereoisomer). Particularly, one or more alanines may be substituted. In the alternative, terminal amino acids may be employed having unnatural chirality. The peptide may also include a terminal amide or a terminal acylated amino acid, particularly acetylated or alkylated, particularly methylated, amino acids. Where a cysteine provides the metal-ion binding domain at the N-terminus, the cysteine may be alkylated or unsubstituted on the mercaptan group.

The YIGSR-containin peptide product may be used to monitor or treat normal or abnormal tissues and metabolic events characterized by accumulation of cells with receptors for YIGSR-containing

peptides, which accumulations will generally produce a photon-abundant area using most imaging modalities, particularly those involving detection of gamma rays. Most commonly, the product will be used to detect accumulations of platelets. There are a number of clinical conditions in which there are platelet accumulations; these include venous thrombosis, arterial thrombosis, left ventricular thrombosis, pulmonary embolism, inflammatory response secondary to myocardial infarction, endocarditis, bypass graft occlusion, aneurysms, prosthetic arterial graft platelet accumulation or occlusion, cerebral embolism or hemorrhage, traumatic injury with hemorrhage, gastrointestinal hemorrhage, and thrombosis secondary to catheters and other implanted devices.

The terms "bind," "binding," "complex," and "complexing," as used throughout the specification and claims, are intended to cover all types of physical and chemical binding, reactions, complexing, attraction, chelating and the like.

The product can be used in a variety of medical procedures including gamma scintigraphy, specific photon emission computerized tomography, positron emission tomography, and magnetic resonance imaging. It is also possible to use the product to deliver a therapeutic quantity of radiation to a disease site. The medical application of the product of this invention depends on the type of peptide and the type of medically useful metal ion used.

In Rhodes BA, U.S. Patent 5,078,985, *Radiolabeling Antibodies and Other Proteins with Technetium or Rhenium by Regulated Reduction*, a process is taught in which disulfide bonds are first partially reduced with stannous salts or other disulfide reducing agents, the resulting combination is purified, and a specified amount of radionuclide reducing agent is added.

In Rhodes BA, U.S. Patent 5,102,990, entitled *Direct Radiolabeling of Antibodies and Other Proteins with Technetium or Rhenium*, a method, product and kit is provided, wherein proteins containing one or more disulfide bonds are radiolabeled with radionuclides for use in diagnosis and treatment of a variety of pathologic conditions. Radiolabeling is accomplished by partial reduction of the disulfide bonds of the protein using Sn (II), or using other reducing agents followed by the addition of Sn (II), removal of excess reducing agent and reduction by-products, and addition of a specified amount of radionuclide reducing agent, such as stannous tartrate, with the addition accomplished in such a manner that further reduction of the protein is limited. The methods and kit of the '275 application are useful in the present invention. The discussions therein pertaining to technetium and rhenium are also

appropriate for the other radiometals and metal ionic forms described herein. Accordingly, the teachings of this application are incorporated herein by reference.

In Rhodes BA and Zamora PO, United States Patent Application Serial No. 07/816,477, entitled *Direct Labeling of Antibodies and Other Proteins with Metal Ions*, a method is taught in which a protein substrate, including peptides, containing monosulfides or disulfide bonds is labeled with a medically useful metal ion by the following method:

a) incubating the protein with a reducing agent to reduce some or all of the disulfide bonds to thiolate groups, or to maintain monosulfides as thiolate groups;

b) removing excess reducing agent from the protein substrate containing thiolate groups;

c) adding a source of Sn (II) agent to the thiolate-containing protein preparation in an amount sufficient to form Sn (II)-containing and sulfur-containing complexes; and,

d) adding a medically useful metal ion whereby the metal ion displaces the Sn (II) in the Sn (II)-containing and sulfur-containing complexes and the metal ion and thiolate-containing protein form metal ion-containing and sulfur-containing complexes.

This invention also teaches that it is possible to chemically modify the protein by the introduction of disulfide bonds. A protein, even though it may not natively contain monosulfides or disulfide bonds, with attached or complexed disulfide bonds can be labeled. The discussions therein pertaining to medically useful metal ions are also appropriate for use with peptides described herein which contain cysteine or penicillamine, and thus contain one or more disulfide bonds or one or more monosulfides. Accordingly, the teachings of this application are incorporated herein by reference.

In Rhodes BA, United States Patent Application Serial No. 07/816,476, entitled *Direct Radiolabeling of Antibody Against Stage Specific Embryonic Antigen for Diagnostic Imaging*, antibody against stage specific embryonic antigen-1 is radiolabeled by direct means with a radionuclide for use in detection of occult abscess and inflammation. Radiolabeling is accomplished by partial reduction of the disulfide bonds of the antibody using Sn(II), or using other reducing agents followed by the addition of Sn(II), removal of excess reducing agent and reduction by-products, and addition of a specified amount of radionuclide reducing agent, such as stannous tartrate. The antibody is specific for human granulocytes, and can be used to image sites of occult abscess and inflammation. Accordingly, the teachings of this application are incorporated herein by reference.

In Rhodes BA, United States Patent Application Serial No. 07/840,076, entitled *Leukostimulatory Agent for In Vivo Leukocyte Tagging*, the use of a variety of leukostimulatory substances, including lectins, peptides and immunoglobulins, labeled or to be labeled with medically useful metal ions, is taught. These teachings, which also involve labeling through disulfide bonds or monosulfides, are specifically applicable to peptides containing cysteine or penicillamine. According, the teachings of that application are incorporated herein by reference.

In Zamora PO and Rhodes BA, United States Patent Application Serial No. 07/840,077, entitled *Peptide-Metal Ion Pharmaceutical Preparation and Method*, the use of peptide-based metal-ion labeled compositions as pharmaceuticals is taught, together with methods of labeling peptides, proteins and other similar substances with radiometals, paramagnetic metals and other medically useful metal ions. This invention also teaches that peptides containing a biological-function domain and a medically useful metal ion-binding domain can be labeled with medically useful metal ions for use in diagnosis and treatment of a variety of pathologic conditions. Specific medically useful metal-ion labeled peptides for detection of thrombus, cancer, infection and inflammation are provided. Accordingly, the teachings of this application are incorporated herein by reference.

In Zamora PO, a United States patent application filed December 30, 1992, entitled *YIGSR Peptide Radiopharmaceutical Applications*, the use of peptides containing a biological-function domain which includes the sequence Tyr-Ile-Gly-Ser-Arg (YIGSR) and a medically useful metal ion-binding domain are labeled with medically useful metal ions for use in a variety of diseases and pathologic conditions, and particularly for diagnosis and treatment of thrombosis and other diseases and conditions. Accordingly, the teachings of this application are incorporated herein by reference.

In Zamora PO, a United States patent application filed December 30, 1992, entitled *IKVAV Peptide Radiopharmaceutical Applications*, the use of peptides useful for lung imaging, and preferably containing a biological-function domain which includes the sequence Ile-Lys-Val-Ala-Val (IKVAV) and a medically useful metal ion-binding domain are labeled with medically useful metal ions for use in a variety of diseases and pathologic conditions, and particularly diagnostic imaging of diseases and pathologic conditions of the lung, is taught. Accordingly, the teachings of that application are incorporated herein by reference.

There are two primary peptide configurations which require

somewhat different methods in order to achieve stable labeling with a metal ion. One peptide configuration involves a metal ion-binding domain which includes one or more disulfide bonds. The most common example of this is

$(R_1)-[Cys-(R_2)-Cys]_n-(R_3)$,

wherein $[Cys-(R_2)-Cys]_n$ is the medically useful metal ion-binding domain, which can appear in the amino acid sequence from 1 time to about 6 times; and R_1, R_2 and R_3 are each amino acid sequences containing from 0 to about 20 amino acids, with at least one of the amino acid sequences R_1, R_2 and R_3 containing the biological-function domain. An example of a peptide fragment meeting this criteria is

... -Phe-Cys-Phe-Trp-Lys-Thr-Cys-Thr- ...

in which the biological-function domain is R_2, being the sequence Phe-Trp-Lys-Thr; the metal ion-binding domain is $[Cys-(R_2)-Cys]_n$, wherein n equals 1, being the sequence Cys-Phe-Trp-Lys-Thr-Cys; R_1 is the sequence ... -Phe; and R_3 is the sequence Thr- ... Other peptide configurations in which reducible disulfide bonds are present are also included in this method. These include the substitution of Pen for one or both Cys amino acids, as well as the modification of a native Met to allow it to form a disulfide bond. The biological-function domain can appear in any one of R_1, R_2 and R_3, and can also span more than one region, so that the biological-function domain may constitute, for example, R_2 and R_3, or some portion of R_2 and R_3. Any one or more of the regions R_1, R_2 and R_3 may contain no amino acids. Examples of peptides which contain disulfide bonds include antibiotic peptides such as defensin HNP-2, atrial natriuretic peptide and its analogues, diabetes-associated peptide, calcitonin, calcitonin gene related peptide, endothelin 1, endothelin 2, endothelin 3, $Pen^{2,5}$-enkephalin, transforming growth factor and related peptides, [Cys^4, Phe^7, Cys^{10}] melalocyte stimulating hormone and its analogues, oxytocin and its analogues, vasopressin and its analogues, somatostatin and its analogues, and substance P analogues which contain cysteine-based disulfide bonds.

In those peptides in which the metal ion-binding domain includes one or more disulfide bonds, it is necessary to first reduce the disulfide bond or bonds. In a preferred embodiment, the following method is employed:

a) incubating the peptide with a reducing agent to reduce some or all of the disulfide bonds to thiolate groups;

b) removing excess reducing agent from the peptide substrate containing thiolate groups;

c) adding a source of Sn (II) agent to the thiolate-containing peptide preparation in an amount sufficient to

form Sn (II)-containing and sulfur-containing complexes; and,

d) adding a medically useful metal ion whereby the metal ion displaces the Sn (II) in the Sn (II)-containing and sulfur-containing complexes and the metal ion and thiolate-containing peptide form metal ion-containing and sulfur-containing complexes.

The order of the steps may be altered, and the method will still produce metal ion-labeled peptides. Accordingly, the claims are not limited to the order of steps presented therein. Specifically, it is possible, and in some cases advantageous, to add the Sn (II) to form Sn (II)-containing and sulfur-containing complexes prior to removing excess reducing agent from the peptide substrate. In this way, oxidation of thiolate groups or reformation of disulfide bonds and other cross-linkages is immediately minimized.

Numerous reducing agents have been described and are known to those skilled in the art. Particularly useful types of reducing agents include 2-mercaptoethanol; 1,4-dithiotheitol; 2,3-dihydroxybutane-1,4-dithiol; 2-aminoethanethiol HCl; 2-mercaptoethylamine; thioglycolate; cyanide; cysteine; reduced glutathione; Sn (II); Cu (I); and Ti (II). The reducing agent may be dissolved in a solute or may be attached to a solid phase. Reducing agents attached to a solid phase are commercially available, and methods for their use are known to those skilled in the art. The degree to which the peptide requires disulfide bond reduction depends on the nature of the peptide and its intended medical application. Generally speaking, milder reduction conditions and shorter incubation periods are required than is required to reduce disulfide bonds in proteins or complex polypeptides, such as antibodies. In any event, reduction is halted before excessive fragmentation of the peptide or loss of the biological-function of the peptide occurs.

In one specific embodiment, Sn (II) is used as a reducing agent at a concentration of 5 mM. In this embodiment the Sn (II) is dissolved in a buffer composed of approximately 10 mM tartrate and 40 mM phthalate, pH 5.5, and the Sn (II) buffer admixed with a peptide substrate at a concentration of 8.3 mg/ml. The reduction reaction is allowed to proceed for a period of time at room temperature, three hours having been employed successfully with some peptides containing a single disulfide bond, after which time the reaction is terminated by removing excess Sn (II) ions by molecular sieve chromatography. One means of molecular sieve chromatography employs Sephadex G-25, with the chromatography gel pre-equilibrated, and the peptide eluted in 0.9% NaCl or other suitable buffer.

Removal of the reducing agent, whether Sn (II) or some other reducing agent, can be accomplished by a variety of suitable means, including such methods as dialysis, ultrafiltration, positive-pressure membrane filtration, precipitation, preparative high performance liquid chromatography, affinity chromatography, other forms of chromatography and preparative isoelectric focusing. Many of the reducing agents contain thiols, which if present in the final labeling mixture, can complex with the medically useful metal ion. Such complexes can have severe and unknown side effects if administered in vivo. Additionally, some reducing agents exhibit unacceptable toxicity. Thus removal of the reducing agent both limits the degree of reduction to that desired, as well as providing for increased utility and safety of the labeled preparation by removal of toxic or otherwise undesirable reducing agents.

Thiolate groups in reduced peptides are highly reactive and can interact to reform disulfide bonds. The use of Sn (II) is believed to minimize the reformation of disulfide bonds. Sources of Sn (II) include stannous tartrate, stannous glucoheptonate, stannous gluconate, stannous phosphonate, stannous chloride, and stannous fluoride. The selection of the source of Sn (II) and its final concentration depends on the intended medical application of the peptide, the nature of the peptide, the relative and absolute number of thiolate groups and the metal ion to be used. In one embodiment stannous tartrate is used at a concentration of 1.25 mM. The stannous tartrate is added to the peptide after removal of the peptide-reducing agent. The stannous tartrate is prepared in a buffer composed of 10 mM tartrate and 40 mM phthalate, pH 5.6, and is added to peptide to yield a final concentration of 1 mg/ml peptide solution.

Sn (II) can be stabilized by use of dicarboxylic acids, such as phthalate and tartrate. A wide range of dicarboxylic acids, known to those skilled in the art, may be similarly used to stabilize the Sn (II) and/or to act as a buffer. If the phthalate and tartrate are in molar excess relative to the Sn (II), then these dicarboxylic acids also stabilize the medically useful metal ion in a form which can react with the peptide. In one embodiment tartrate and phthalate are used in the Sn (II) agent at concentrations of 10 mM and 40 mM, respectively.

Similarly, the Sn (II) and the medically useful metal ion may be stabilized by free amino acids used singly or in combination with other agents. The type of amino acid used and the specific concentration depends on the nature of the peptide and its intended use. In one embodiment, glycine is used at a concentration of 0.1-10 mM, and in another, histidine is used at a concentration of 0.1-10

mM.

The peptide may be stored frozen in bulk form after disulfide bond reduction and the removal of excess reducing agent. Alternatively, the peptide may be stored in bulk form or in unit dose form after addition of the Sn (II). Similarly, the peptide may be stored lyophilized during or after processing. For example, in one embodiment the peptide is stored in vials after introduction of the Sn (II). Methods used in lyophilization of peptides are known to those skilled in the art. Either frozen or lyophilized preparations may be maintained for an indefinite period before labeling by the addition of the medically useful metal ion.

In both the frozen and lyophilized storage forms, excipients may be added to the peptide to minimize damage which can arise from ice-crystal formation or free-radical formation. The type of excipient and the concentration depends on the nature of the peptide and the intended use. In one embodiment, glycine and inositol are used as excipients in lyophilized preparations.

A typical lyophilized preparation made by the embodiments set forth above would, upon rehydration, contain approximately 10 mM tartrate, 40 mM phthalate, 22 µg of Sn (II), 500 µg of peptide, 2 mg/ml of glycine, and 2 mg/ml of inositol. The amounts of peptide and Sn (II) used in the kits would depend on the medical application, varying depending on biodistribution of the peptide, imaging modality being used, type of metal ion and related factors. Similarly, the amount and type of buffer components (such as tartrate and phthalate) and excipients (such as glycine and inositol) depends on the specific application.

To label with a medically useful metal ion, a typical lyophilized preparation is hydrated by the addition of a solution containing 0.9% NaCl (U.S.P.) or water for injection (U.S.P.) and the medically useful metal ion. Alternatively, it is possible to hydrate the lyophilized preparation, and to add the metal ion in a subsequent step. If a frozen preparation is used, it is thawed and allowed to come to room temperature, and a solution containing the medically useful metal ion is then added. The nature and amount of the medically useful metal ion and the specific reaction conditions depend on the isotopic nature of the metal, and the intended medical application. In one embodiment, ^{99m}Tc is added in the form of pertechnetate ion in a solution of 0.9% NaCl. The ^{99m}Tc is typically incubated for up to 30 minutes to insure completion of the reaction with the peptide, after which the radiolabeled preparation can be directly used in medical applications. In another embodiment, ^{67}Cu is added in a solution of 10 mM tartrate and 40 mM phthalate at pH 5.6.

In yet another embodiment, ^{186}Re or ^{186}Re is added to a solution of 10 mM tartrate and 40 mM phthalate, at pH 5.6, and containing Sn (II), and then heated to lower the oxidation state of Re. The resulting solution is then added to the lyophilized or frozen preparation.

In the embodiment in which ^{99m}Tc is used, the Sn (II) is present in the peptide-containing solution in sufficient excess to alter the oxidation state of the Tc ion such that it can bind to thiolate groups. Typically Tc (VII) is reduced to Tc (III), Tc (VI), and/or Tc (V). The preferred state of Tc to be added to peptide preparations is as the pertechnetate ion, $(TcO_4)^-$. The Sn (II) then reacts with the pertechnetate ion resulting in a lower oxidation state in which the Tc is reactive with thiolate groups. Similar approaches may be used to lower the oxidation state of other medically useful metal ions for subsequent binding to thiolate groups. The type of the metal ion, its isotopic nature, and concentration would depend on the intended medical application.

The other peptide configuration involves one or more amino acids containing sulfur, nitrogen or oxygen which is available for binding, or which can be made available for binding to metal ions. Commonly used amino acids include Cys, Pen and His, or any combination of them. This peptide configuration does not involve initial reduction of disulfide bonds. The simplest case takes the form

$$(R_1)-[Cys]_n-(R_2)$$

wherein $[Cys]_n$ is the medically useful metal ion-binding domain and n is typically a number between 1 and about 6; and R_1 and R_2 are each an amino acid sequence containing from 0 to about 20 amino acids, with at least R_1 and R_2 including the biological-function domain. In this and all related forms, it should be noted that R_1 and R_2 are interchangeable; either can contain the biological-function domain, the biological-function domain may include part or all of both R_1 and R_2, and the biological-function domain may constitute only a portion of the amino acid sequence in either R_1 or R_2. The order of components for these purposes can be varied, so that $(R_1)-[Cys]_n-(R_2)$, $(R_2)-[Cys]_n-(R_1)$, $[Cys]_n-(R_2)-(R_1)$, $[Cys]_n-(R_1)-(R_2)$ and the mirror images of the last two orderings are all equivalent, even though the resulting peptides may significantly differ in other aspects. A representative example of this form is the sequence

Cys-Asp-Pro-Gly-Try-Ile-Gly-Ser-Arg

in which the Cys is $[Cys]_n$ wherein n is 1, Tyr-Ile-Gly-Ser-Arg is the biological-function domain (R_1) and Asp-Pro-Gly is (R_2), so that the structure of the sequence is $[Cys]_n-(R_2)-(R_1)$.

Other forms of the same general configuration include

$(R_2)-[Cys-(R_2)-Cys]_n-(R_3)$,

$(R_1)-[Cys-(R_2)-Pen]_n-(R_3)$,
$(R_1)-[His-(R_2)-Cys]_n-(R_3)$,
$(R_1)-[His-(R_2)-Pen]_n-(R_3)$,
and $(R_1)-[His-(R_2)-His]_n-(R_3)$

wherein the sequence $[...]_n$ is the medically useful metal ion-binding domain with n typically being a number between 1 and about 6; and R_1, R_2 and R_3 are each an amino acid sequence containing from 0 to about 20 amino acids, with at least one of R_1, R_2 and R_3 including the biological-function domain. Here too the ordering is irrelevant to the functional description; for example, $(R_3)-[His-(R_2)-Cys]_n-(R_1)$, $(R_1)-(R_3)-[His-(R_2)-Cys]_n$, $(R_3)--(R_1)-[His-(R_2)-Cys]_n$, mirror images of the foregoing two orderings, all orderings in which the positions of His and Cys are reversed, and orderings in which the biological-function domain is present in the any of the three regions R_1, R_2 and R_3, any portion of the three regions R_1, R_2 and R_3, or any combination of the three regions R_1, R_2 and R_3, are all equivalent to the third configuration listed above, $(R_1)-[His-(R_2)-Cys]_n-(R_3)$. Each of the other foregoing configurations can be similarly described.

In one preferred embodiment of the method for labeling peptides of the configurations set forth above, the following method can be employed:

a) adding a source of positively-charged transition metal, most preferably an Sn (II) agent, to the peptide containing amino acids comprising sulfur, nitrogen or oxygen which is available for binding, or which can be made available for binding to metal ions, in an amount sufficient to allow the positively-charged transition metal to undergo a replacement reaction, thereby forming transition metal-containing and sulfur-, nitrogen- or oxygen-containing complexes, or some combination thereof; and,

b) adding a medically useful metal ion whereby the metal ion displaces the transition metal in the transition metal-containing and sulfur-, nitrogen- or oxygen-containing complexes and the metal ion and peptide form metal ion-containing and sulfur-, nitrogen-, or oxygen-containing complexes.

The preferred transition metal is Sn (II); useful sources of Sn (II) include stannous tartrate, stannous glucoheptonate, stannous gluconate, stannous phosphonate, stannous chloride, and stannous fluoride. The selection of the source of Sn (II) and its final concentration depends on the intended medical application of the peptide, the nature of the peptide, the relative and absolute number of thiolate groups and the metal ion to be used. In one embodiment stannous tartrate is used at a concentration of 1.25 mM. The

stannous tartrate is prepared in a buffer composed of 10 mM tartrate and 40 mM phthalate, pH 5.6, and is added to peptide to yield a final concentration of 1 mg/ml peptide solution.

As is the case in the method involving reduction of disulfide bonds, Sn (II) can be stabilized by use of dicarboxylic acids, such as phthalate and tartrate. A wide range of dicarboxylic acids, known to those skilled in the art, may be similarly used to stabilize the Sn (II) and/or to act as a buffer. If the phthalate and tartrate are in molar excess relative to the Sn (II), then these dicarboxylic acids also stabilize the medically useful metal ion in a form which can react with the peptide. In one embodiment tartrate and phthalate are used in the Sn (II) agent at concentrations of 10 mM and 40 mM, respectively.

Similarly, the Sn (II) and the medically useful metal ion may be stabilized by free amino acids used singly or in combination with other agents. The type of amino acid used and the specific concentration depends on the nature of the peptide and its intended use. In one embodiment, glycine is used at a concentration of 0.1-10 mM, and in another, histidine is used at a concentration of 0.1-10 mM.

The peptide may be stored in bulk form or in unit dose form after addition of the Sn (II) or other transition metal. For example, in one embodiment the peptide is stored at -20°C in vials after introduction of the Sn (II). Methods used in lyophilization of peptides are known to those skilled in the art. Either frozen or lyophilized preparations may be maintained for an indefinite period before labeling by the addition of the medically useful metal ion.

In both the frozen and lyophilized storage forms, excipients may be added to the peptide to minimize damage which can arise from ice-crystal formation or free-radical formation. The type of excipient and the concentration depends on the nature of the peptide and the intended use. In one embodiment, glycine and inositol are used as excipients in lyophilized preparations.

A typical lyophilized preparation made by the embodiments set forth above would, upon rehydration, contain 10 mM tartrate, 40 mM phthalate, 22 µg of Sn (II), 500 µg of peptide, 2 mg/ml of glycine, and 2 mg/ml of inositol. To label with a medically useful metal ion, a typical lyophilized preparation is hydrated by the addition of a solution containing 0.9% NaCl (U.S.P.) or water for injection (U.S.P.) and the medically useful metal ion. Alternatively, it is possible to hydrate the lyophilized preparation, and to add the metal ion in a subsequent step. If a frozen preparation is used, it is

thawed and allowed to come to room temperature, and a solution containing the medically useful metal ion is then added. The nature and amount of the medically useful metal ion and the specific reaction conditions depend on the isotopic nature of the metal, and the intended medical application. In one embodiment, ^{99m}Tc is added in the form of pertechnetate ion in a solution of 0.9% NaCl. The ^{99m}Tc is typically incubated for up to 30 minutes to insure completion of the reaction with the peptide, after which the radiolabeled preparation can be directly used in medical applications. In another embodiment, ^{67}Cu is added in a solution of 10 mM tartrate and 40 mM phthalate at pH 5.6. In yet another embodiment, ^{188}Re or ^{186}Re is added to a solution of 10 mM tartrate and 40 mM phthalate, at pH 5.6, and containing Sn (II), and then heated to lower the oxidation state of Re. The resulting solution is then added to the lyophilized or frozen preparation.

In the embodiment in which ^{99m}Tc is used, the Sn (II) is present in the peptide-containing solution in sufficient excess to alter the oxidation state of the Tc ion such that it can bind to ionizable groups. Similar approaches may be used to lower the oxidation state of other medically useful metal ions for subsequent binding to ionizable groups. The type of the metal ion, its isotopic nature, and concentration would depend on the intended medical application.

It is also possible to administer a peptide, such as the YIGSR-containing or IKVAV-containing peptide, and to perform the actual radiolabeling in vivo. This can be done, for example, using a biotin-avidin system, in which biotin is conjugated to the YIGSR-containing or IKVAV-containing peptide, which is then injected into the patient. A radioisotope-labeled avidin complex is then injected, which binds to the peptide-biotin complex, forming a peptide-biotin-avidin-radiolabel complex, which can be detected by gamma scintigraphy or other detection means. This method presents certain advantages, in that maximum clearance and target binding parameters can be attained. To use this system, for example, it is possible to employ Biotin-HPDP (Pierce Chemical Co.), a cleavable, sulfhydryl-reactive biotinylation reagent. The IKVAV-containing peptide is dissolved in a 100 mM borate buffer pH 8.0 to a final concentration of 1 mg/ml, and biotin-HPDP at 1 mg/ml is added. The solution is mixed and incubated for 1 hour, and the biotinylated peptide separated from unconjugated materials by molecular sieve chromatography over Sephadex G-25. Avidin or strepavidin can be directly iodinated with ^{131}I by standard methods. Alternatively, avidins can be conjugated to chelating agents such as DTPA or other agents which introduce thiols into the protein, and radiolabeled with ^{99m}Tc. For use in vivo, the biotinylated peptide is injected intravenously and allowed to localize and clear from the general

circulation, a time period generally of from 1 to 2 hours. Radiolabeled avidin is then injected; the radiolabeled avidin binds to the biotin, and consequently localizes the disease lesion.

The invention is further illustrated by the following non-limiting examples.

EXAMPLE 1 - DISULFIDE BOND CONTAINING PEPTIDE

Phe-Cys-Phe-Trp-Lys-Thr-Cys-Thr-ol

The peptide is a cyclic octapeptide analogue of somatostatin. The biological-function portion of the molecule is associated with the Phe-Trp-Lys-Thr portion of the molecule. The disulfide bridge between the two cysteine residues is reduced using an Sn (II) reducing agent, presumptively forming sulfur-tin complexes. The peptide was obtained in acetate buffer pH 4.4. To the peptide containing solution was added (1:1) 10 mM tartrate/40 mM phthalate buffer, pH 5.6 (P/T buffer), to result in a solution containing 500 µg of peptide/ml. This solution was mixed (1:1) with P/T buffer containing 1.25 mM stannous tartrate, and allowed to incubate at room temperature for at least three hours. Aliquots of 0.5 ml were then dispensed into individual vials. Each kit contained 0.25 mg of peptide, 40 mM phthalate, 10 mM tartrate, and 44 µg of stannous tartrate. All solutions were purged with nitrogen prior to use and all preparations made under an anaerobic atmosphere. The peptide in the labeling kits was labeled with ^{99m}Tc by addition of 1-2 mCi of sodium pertechnetate (U.S.P.) and allowing the reaction to proceed for 30 minutes.

EXAMPLE 2 - CHEMOTATIC PEPTIDE ANALOGUE

N-formyl-Met-Leu-Phe-Gly-His-Gly-Gly-His-Gly-His-Gly-Gly-His

This peptide is a chemotactic peptide analogue, specifically an analogue of N-formyl-Met-Leu-Phe, one of several peptides which are chemotactic for cells of the lymphatic system. The sequence His-Gly-Gly-His-Gly-His-Gly-Gly-His is used to bind Tc. The peptide was dissolved directly in 10 mM tartrate/40 mM phthalate buffer, pH 5.6 (P/T buffer), to result in a solution containing 1.4 mg of peptide/ml. This solution was mixed (7:3) with P/T buffer containing 1.25 mM stannous tartrate. Aliquots of 0.5 ml was then dispensed into individual vials. Each kit contained 0.5 mg of peptide, 40 mM phthalate, 10 mM tartrate, and 22 µg of stannous tartrate. All solutions were purged with nitrogen prior to use and all preparations

prepared under an anaerobic atmosphere. The peptides in the labeling kits were labeled with ^{99m}Tc by addition of 1-2 mCi of sodium pertechnetate (U.S.P.) and allowing the reaction to proceed for 30 minutes.

EXAMPLE 3 - COMPARATIVE POTENTIAL BINDING STUDY

To compare the potential binding of ^{99m}Tc to histidine and cysteine, ^{99m}Tc binding in three peptides with known amino acid sequences was evaluated. One peptide, with the amino acid sequence H_2N-Cys-Asp-Pro-Gly-Tyr-Ile-Gly-Ser-Arg, contained a single cysteine residue and no histidines, and was prepared by dissolving directly in 10 mM tartrate/40 mM phthalate buffer, pH 5.6 (P/T buffer), resulting in a solution containing 1.4 mg of peptide/ml. This solution was mixed (7:3) with P/T buffer containing 1.25 mM stannous tartrate. Aliquots of 0.5 ml was then dispensed into individual vials. Each vial contained 0.5 mg of peptide, 40 mM phthalate, 10 mM tartrate, and 22 µg of stannous tartrate. All solutions were purged with nitrogen prior to use and all preparations prepared under an anaerobic atmosphere. The peptide in the vials was labeled with ^{99m}Tc by addition of 1-2 mCi of sodium pertechnetate (U.S.P.) and allowing the reaction to proceed for 30 minutes.

Another peptide, with the sequence (Acetyl)-Asp-Arg-Val-Ile-His-Pro-Phe-His-Leu-Val-Ile-His-Asp, contained histidine residues but no cysteines or cystine, and was prepared and radiolabeled as set forth in Example 2. The control, poly-tyrosine, contained neither histidine nor cysteine; it was prepared and radiolabeled as set forth in the preceding paragraph.

The histidine-containing peptide bound some but not all the added ^{99m}Tc. The cysteine-containing peptide bound essentially all of the added ^{99m}Tc. Poly-tyrosine, the negative control material, did not label. These results were confirmed by conventional thin-layer chromatography.

EXAMPLE 4 - SYNTHESIS OF PEPTIDE CONTAINING BIOLOGICAL-FUNCTION DOMAIN AND METAL ION-BINDING DOMAIN

The chemotactic peptide analogue N-formyl-Met-Leu-Phe-Gly-His-Gly-Gly-His-Gly-His-Gly-Gly-His was synthesized using a commercially available automated synthesizer. The peptide was lyophilized and purified using reverse phase HPLC. The peptide was then labeled using the methods of Example 2.

EXAMPLE 5 - PREPARATION OF IKVAV-CONTAINING PEPTIDE KITS FOR ^{99m}Tc LABELING

Laminin-derived peptide of the sequence CSRARKQAASIKVAVSADR was obtained commercially (Bachem, Inc.) as lyophilized powder and used

without additional purification. The N-terminal thiolate associated with the Cys residue was used as the metal ion-binding domain for subsequent labeling with reduced ^{99m}Tc.

Peptide labeling kits were prepared aseptically using nitrogen-purged solutions, and whenever feasible under an atmosphere of nitrogen. To prepare the peptide labeling kits, the peptide was dissolved to a final concentration of 1.4 mg/ml in chilled, nitrogen-purged 10 mM tartrate/40 mM phthalate buffer, pH 5.6 (P/T buffer) containing 2% maltose. The peptide and P/T buffer solution was then mixed (7:3) with P/T buffer containing 1.25 mM stannous tartrate. Aliquots (typically 0.5 ml containing 500 µg of peptide) were then sterile filtered through a 0.22 micron filter, and dispensed into individual vials. The head space of each vial was purged with nitrogen, the vials stoppered and crimped, and stored frozen at -70°C.

EXAMPLE 6 - ^{99m}Tc LABELING OF IKVAV-CONTAINING PEPTIDE KITS

To radiolabel, a vial of the preparation of Example 5 was removed from the freezer and allowed to come to room temperature. The labeling reaction was initiated by the addition of 0.5 - 2.0 mCi of ^{99m}Tc (sodium pertechnetate in saline). Radiochemical analysis was begun 30 minutes after the introduction of the pertechnetate.

EXAMPLE 7 - RADIOCHEMICAL ANALYSIS BY CHROMATOGRAPHY OF IKVAV-CONTAINING PEPTIDE KITS

To determine the relative amount of ^{99m}Tc bound to a given peptide preparation of Example 5, aliquots of the ^{99m}Tc-labeled preparations made by the method of Example 6 were analyzed by molecular sieve HPLC, reverse phase chromatography, and thin layer chromatography.

Molecular sieve HPLC was performed using a 7.5 x 300 mm TSK G3000SW column preceded with a TSK-SW 7.5 x 7.5 mm guard column (TosoHaas, Philadelphia, PA) at a flow rate of 1 ml/minute of a phosphate buffered saline solution (0.01 M phosphate, pH 7.0, containing 0.15 M NaCl), with a UV and radioisotope detector in series. The ^{99m}Tc-IKVAV-containing peptide preparation eluted at 12.8 minutes with a low chromatographic recovery (less than 10%). In control studies, pertechnetate eluted at 17.8 minutes with essentially quantitative chromatographic recovery.

For reverse-phase analysis, Sep-Pak C_{18} mini-columns (Millipore Inc., Bedford, MA) were used as reverse-phase adsorbents to evaluate the binding of ^{99m}Tc to the peptides. The columns were rinsed with 10 ml of 100% ethanol followed by 10 ml of 0.001% HCl. Aliquots of 100 µl of the test sample were loaded onto the column and the unbound

material eluted with 10 ml of 0.001% HCl. The column was then serially eluted with a graded series of 10 ml solutions of aqueous ethanol (10%, 20%, 30%, 40%, 50%, 60%, and 100%). The radioactivity in each eluant fraction (0.001% HCl through 100% ethanol) was determined by counting an aliquot (20 µl) of each fraction in a gamma scintillation counter. The columns themselves were also counted, after allowing an appropriate time for decay. All counts were corrected for decay and the amounts of radioactivity in each fraction expressed as a percentage of the total radioactivity assayed. The reverse-phase chromatography using C_{18} mini-columns eluted with a graded series of ethanol confirmed ^{99m}Tc binding to the peptide (Table 1).

TLC was used to measure the amount of peptide-bound (and unbound) ^{99m}Tc and the amount of radiolabeled aggregate/colloid. Both measurements involved the use of ITLC-SG (Gelman Sciences, #61886) chromatography paper, cut into 1.5 x 10 cm strips and activated by heating for 30 minutes at 110°C, as per the manufacturer's instructions. After heating, the strips were stored at room temperature until use.

Peptide-bound ^{99m}Tc in the radiolabeled preparations was measured using TLC in 85% aqueous methanol using ITLC-SG strips. The solvent separated the soluble, unbound ^{99m}Tc (which migrates with the solvent front) from ^{99m}Tc bound to the peptide (which remains at the origin). Percentage of unbound ^{99m}Tc was expressed as CPM in the origin half of the strip divided by the total CPM, with all measures corrected for background.

Thin layer chromatography of the ^{99m}Tc-IKVAV-containing peptide preparation of Example 5 in saline over heat-activated silica-gel coated cellulose (ITLC-SG paper) showed essentially all radioactivity associated with the peptide (R_f = 0). The preparations did not contain significant amounts of unbound ^{99m}Tc as pertechnetate or ^{99m}Tc-tartrate (R_f = 1.0).

TABLE 1. Elution of ^{99m}Tc-IKVAV-containing peptide preparation of Example 5 from C_{18} reverse-phase columns by increasing concentrations of ethanol. Tartrate was used in the kits as a ^{99m}Tc transfer agent. In the absence of peptide tartrate retains ^{99m}Tc, and its elution is provided here as a reference.

Percent EtOH in Eluent	PERCENTAGE OF TOTAL RADIOACTIVITY ASSAYED	
	^{99m}Tc-Tartrate	^{99m}Tc-Peptide
0 %	90.9 %	0.8 %
10 %	2.2 %	0.9 %
20 %	1.6 %	0.2 %
30 %	0.8 %	0.3 %
40 %	0.5 %	0.5 %
50 %	0.8 %	0.3 %
60 %	0.5 %	0.3 %
100 %	1.1 %	0.4 %
On Column	1.6%	95.7 %

EXAMPLE 8 - BIODISTRIBUTION IN RODENTS OF IKVAV-CONTAINING ^{99m}Tc-PEPTIDE

The biodistribution of the ^{99m}Tc-peptide of Example 5 was evaluated in adult female Swiss-Webster mice (approximately 19 g) at selected times (10, 30, and 120 minutes) after injection into the tail vein. Each experimental group was composed of at least five animals, with each animal receiving 0.1 ml containing 5 µg of peptide (1 µCi/µg). Animals were sacrificed by Halothane overdose, and selected organs dissected, weighed, and associated radioactivity determined. Data were analyzed using a computer program specifically designed for ^{99m}Tc-labeled preparations. The percent dose per organ for blood, bone, and muscle were calculated assuming 7, 8.2, and 40% of total body weight, respectively, for these tissues.

Following the injection of ^{99m}Tc-IKVAV-containng peptide of Example 5, a significant amount of radioactivity was found in the lungs at both 10 and 30 minutes post injection (Table 2). Major accumulations were also found in the liver and kidneys. By two hours post injection the amount of radiolabel in the lung had fallen to less than 5% (from 47% at 10 minutes post injection, with a concomitant increase in kidney activity noted). Only small uptakes of ^{99m}Tc were noted in other organs. At 10 and 30 minutes post injection the lung-to-blood ratios for ^{99m}Tc-peptide of Example 1 were 21:1 and 23:1, respectively.

TABLE 2. Biodistribution of ^{99m}Tc-IKVAV-containing peptide of Example 5 in normal Swiss-Webster mice at selected times after injection. All values are the mean ± standard deviation. n=6 for all data points.

	% INJECTED DOSE/ORGAN		
ORGAN	10 MINUTES	30 MINUTES	120 MINUTES
blood	10.5 ± 1.0	7.6 ± 1.0	6.9 ± 1.7
stomach	0.5 0.1	0.7 0.2	0.6 0.1
sm. intestine	1.4 0.1	2.3 0.5	3.5 0.1
appendix	0.2 0.0	0.2 0.0	1.4 0.3
lg. intestine	0.3 0.1	0.2 0.1	0.9 0.3
liver	14.3 1.4	17.3 1.9	27.5 5.7
spleen	2.2 0.5	3.1 0.3	2.1 5.7
kidneys	7.4 0.6	12.2 1.7	25.1 5.7
heart	0.6 0.3	0.4 0.1	0.1 0.3
lungs	47.4 3.7	27.9 3.7	2.2 0.6
bone	2.7 0.5	2.7 0.2	2.7 0.6
muscle	7.0 2.5	5.9 0.7	3.7 0.8
thyroid	0.1 0.0	0.1 0.0	0.1 0.0

Some studies involved pre-incubation of the ^{99m}Tc-IKVAV-containing peptide in whole blood prior to injection and determination of biodistribution. In these studies, whole human blood was obtained from a healthy adult male donor and collected into Vacutainer tubes containing EDTA. After mixing to insure proper dissolution of the EDTA, approximately 2.5 ml of the whole blood was removed and mixed with 0.25 ml of ^{99m}Tc-peptide. The mixture was allowed to incubate 30 minutes at room temperature. After 30 minutes, aliquots of 0.1 ml were injected into the tail vein of the mice. The amount of radioactivity in the circulation for ^{99m}Tc-IKVAV-containing peptide pre-incubated in whole blood was higher than in animals receiving ^{99m}Tc-IKVAV-containing peptide without incubation in blood. With incubation of ^{99m}Tc-peptide in whole blood prior to injection, significantly decreased lung uptake was noted (Table 3).

TABLE 3. Biodistribution in normal Swiss-Webster mice of ^{99m}Tc-IKVAV-peptide of Example 5 after a 30 minute pre-incubation in whole blood. All values are the mean ± standard deviation. n=6 for all data points.

ORGAN	% INJECTED DOSE/ORGAN 10 MINUTES	30 MINUTES
blood	19.0 ± 1.6	10.8 ± 0.9
stomach	0.6 0.1	0.4 0.1
sm. intestine	2.8 0.2	4.2 0.8
appendix	0.2 0.1	0.1 0.0
lg. intestine	0.4 0.1	0.3 0.0
liver	21.8 1.0	16.9 0.6
spleen	1.0 0.1	1.2 0.2
kidneys	8.1 0.9	8.4 0.9
heart	0.6 0.2	0.4 0.1
lungs	5.1 0.9	3.9 0.3
bone	4.6 0.6	4.3 0.6
muscle	10.5 1.0	6.6 1.4
thyroid	0.1 0.1	0.1 0.0

The clearance rates of the ^{99m}Tc-IKVAV-containing peptide of Example 5 was evaluated in adult female Fischer 344 rats at 2 hours after injection. Each experimental group was composed of three animals. Each animal was anesthetized with ketamine and the bile duct and bladder were cannulated. Blood was collected over various periods of time from the jugular vein. The ^{99m}Tc-peptide cleared very rapidly from the plasma of rats, with a clearance rate of 2.4 ml/minute. At two hours, 10.8 ± 4.9 % of the injected dose had cleared through urine, while bile clearance at the same time point was 0.9 ± 0.3%.

EXAMPLE 9 - DOSE RESPONSE OF ^{99m}Tc-IKVAV PEPTIDE ON LUNG LOCALIZATION

The effect of the dose of ^{99m}Tc-IKVAV-containing peptide of Example 5 on lung localization was evaluated. Localization in the lung was found in all injection doses used (0.05, 0.5, and 5 µg) at both 10 and 30 minutes post injection. Similar amounts of radioactivity were found in the lungs regardless of the amount injected at 10 minutes post injection (110.6%, 111.9%, and 144.4% I.D./gram of lung tissue for 0.05, 0.5, and 5 µg, respectively). At 30 minutes post injection a more pronounced effect of dose was noted, with more radioactivity retained in the lung with a larger injection dose (48.8%, 68.1%, and 91.9% I.D./gram of lung tissue for 0.05, 0.5,

and 5 µg, respectively).

EXAMPLE 10 - EXAMINATION OF IKVAV-CONTAINING PEPTIDE KITS FOR PARTICULATE IMPACTION

Certain experiments were conducted to determine if high lung uptake resulted from the preparation of Example 5 forming a particle which impacts in the lung. No particles were visible when freshly radiolabeled ^{99m}Tc-IKVAV-peptide of Example 5 was examined under a phase contrast microscope, and ^{99m}Tc-IKVAV-peptide of Example 5 filtered through a submicron filter (0.2 micron pore size) still localized to the lungs. Additionally, when ^{99m}Tc-IKVAV-peptide of Example 5 was injected into the peritoneal cavity of mice (to sequester potential colloid), localization to the lung was still found. In these experiments, the lung-heart ratio of ^{99m}Tc-peptide of Example 5 was elevated (3:1) at 15 minutes after i.p. injection, and increased so that by 60 minutes post injection the ratio was 9:1. At 120 minutes the ratio had decreased, but was still nearly 6:1.

EXAMPLE 11 - BIODISTRIBUTION IN MELANOMA TUMOR BEARING MICE OF ^{99m}Tc-IKVAV-CONTAINING PEPTIDE

Biodistribution studies involving nude mice bearing melanoma tumors in the lung were conducted. Aliquots of B-16 melanoma cells were injected (50,000 cells in 0.1 ml serum-free RPMI medium) into the tail vein of adult nude mice and were used in studies approximately 3 weeks after inoculation. Paired studies were done using nude mice receiving sham injections of saline without cells.

The biodistribution of ^{99m}Tc-IKVAV-peptide of Example 5 was markedly altered in animals with tumors in the lung compared to those without tumors in the lung. In these studies, five tumored and five control animals were used for each time point, and results are ± the standard deviation. In tumored animals, the amount of lung uptake (% injected dose) was increased compared to controls at all time points examined, so that with tumored animals lung uptake was 38.3 ± 4.0 %, 28.1 ± 3.6 % and 3.9 ± 1.2 % at 10, 30, and 120 minutes post-injection, while with control nude mice at the same time points, the lung uptake was 19.0 ± 2.7 %, 11.9 ± 2.9 % and 1.6 ± 0.4 %, respectively.

EXAMPLE 12 - BIODISTRIBUTION IN EMPHYSEMA MODEL MICE OF ^{99m}Tc-IKVAV-CONTAINING PEPTIDE

Biodistribution was also studied in a lung disease model which used tight-skin mice with genetic emphysema. The tight-skin (*Tsk*) mouse is a genetic mutant caused by a dominant gene deficiency of serum anti-elastase. Heterozygous (*Tsk/+*) animals show multiple skin connective tissue abnormalities resembling scleroderma as well as an increased growth of cartilage, bone, and small tendons with hyperplasia of the tendon sheaths. The *Tsk* trait is associated with

progressive pulmonary emphysema and development of right ventricular hypertrophy, as well as with lung collagen changes. These mice, as well as genetic control mice (pallid), were obtained from The Jackson Laboratory (Bar Harbor, ME).

The relative localization of ^{99m}Tc-IKVAV-peptide of Example 5 at all time points examined (10, 30, and 120 minutes post-injection), was decreased in the lungs of animals with emphysema relative to paired control animals.

TABLE 4. Biodistribution of ^{99m}Tc-IKVAV-containing peptide of Example 5 in tight-skin (*Tsk*) mice and genetic control mice at selected times after injection. All values are the mean ± standard deviation. n=4 for all time points except 10 minutes, where n=3.

	% INJECTED DOSE/LUNG		
MICE	10 MINUTES	30 MINUTES	120 MINUTES
Tight-skin (*Tsk*) Mice	10.4 ± 2.1	8.8 ± 0.6	8.5 ± 0.8
Genetic Control Mice	15.8 ± 2.7	11.9 ± 0.7	12.8 ± 2.7

In a degenerative lung disease like emphysema the total number of receptors would be expected to decrease, due to loss of lung mass. In such a case, the amount of localization of ^{99m}Tc-IKVAV peptide in the lungs would decrease relative to the localization found in paired genetic control animals. The observations made correlate with this hypothesis.

EXAMPLE 13 - PREPARATION OF YIGSR-CONTAINING PEPTIDE KITS FOR ^{99m}Tc LABELING

Laminin-derived peptide of the sequence CDPGYIGSR (H_2N-Cys-Asp-Pro-Gly-Tyr-Ile-Gly-Ser-Arg) was obtained commercially (Bachem, Inc.) as lyophilized powder and used without additional purification. The N-terminal thiolate associated with the Cys residue was used as the metal ion-binding domain for subsequent labeling with reduced ^{99m}Tc.

Peptide labeling kits were prepared aseptically using nitrogen-purged solutions, and whenever feasible under an atmosphere of nitrogen. To prepare the peptide labeling kits, the peptide was dissolved to a final concentration of 1.4 mg/ml in chilled, nitrogen-purged 10 mM tartrate/40 mM phthalate buffer, pH 5.6 (P/T buffer) containing 2% maltose. The peptide and P/T buffer solution was then mixed (7:3) with P/T buffer containing 1.25 mM stannous tartrate. Aliquots (typically 0.5 ml containing 500 µg of peptide) were then sterile filtered through a 0.22 micron filter, and dispensed into individual vials. The head space of each vial was purged with nitrogen, the vials stoppered and crimped, and stored frozen at -70°C.

EXAMPLE 14 - ^{99m}Tc LABELING OF YIGSR-CONTAINING PEPTIDE KITS

To radiolabel, a vial of the preparation of Example 13 was removed from the freezer and allowed to come to room temperature. The labeling reaction was initiated by the addition of 0.5 - 2.0 mCi of ^{99m}Tc (sodium pertechnetate in saline). Radiochemical analysis was begun 30 minutes after the introduction of the pertechnetate.

EXAMPLE 15 - RADIOCHEMICAL ANALYSIS BY CHROMATOGRAPHY OF ^{99m}Tc-LABELED YIGSR CONTAINING PEPTIDE

To determine the relative amount of ^{99m}Tc bound to a given YIGSR-containing peptide preparation, aliquots of the ^{99m}Tc-labeled preparations of Example 14 were analyzed by molecular sieve HPLC, reverse phase chromatography, and thin layer chromatography.

Molecular sieve HPLC was performed using a 7.5 x 300 mm TSK G3000SW column preceded with a TSK-SW 7.5 x 75 mm guard column (TosoHaas, Philadelphia, PA) at a flow rate of 1 ml/minute phosphate buffered saline (0.01 M phosphate, pH 7.0, containing 0.15 M NaCl), with a UV and radioisotope detector in series. The preparation of Example 13, labeled by the method of Example 14, eluted at 13.9 minutes with a high chromatographic recovery (greater than 95%). In control studies, pertechnetate eluted at 17.8 minutes with essentially quantitative chromatographic recovery.

For reverse-phase analysis, Sep-Pak C_{18} mini-columns (Millipore Inc., Bedford, MA) were used as reverse-phase adsorbents to evaluate the binding of ^{99m}Tc to the peptides. The columns were rinsed with 10 ml of 100% ethanol followed by 10 ml of 0.001% HCl. Aliquots of 100 µl of the test sample were loaded onto the column and the unbound material eluted with 10 ml of 0.001% HCl. The column was then serially eluted with a graded series of 10 ml solutions aqueous ethanol (10%, 20%, 30%, 40%, 50%, 60%, and 100%). The radioactivity in each eluant fractions (0.001% HCL - 100% ethanol) was determined by counting an aliquot (20 µl) of each fraction in a gamma scintillation counter. The columns themselves were also counted, after allowing an appropriate time for decay. All counts were corrected for decay and the amounts of radioactivity in each fraction expressed as a percentage of the total radioactivity assayed. Reverse-phase chromatography using C_{18} mini-columns eluted with a graded series of ethanol confirmed ^{99m}Tc binding to the peptides (Table 5).

TLC was used to measure the amount of peptide-bound (and unbound) ^{99m}Tc and the amount of radiolabeled aggregate/colloid. Both measurements involved the use of ITLC-SG (Gelman Sciences, #61886) chromatography paper, cut into 1.5 x 10 cm strips and activated by

heating for 30 minutes at 110°C, as per the manufacturer's instructions. After heating, the strips were stored at room temperature until use.

Peptide-bound ^{99m}Tc in the radiolabeled preparations was measured using TLC in 85% aqueous methanol using ITLC-SG strips. The solvent separated the soluble, unbound ^{95m}Tc (which migrates with the solvent front) from ^{99m}Tc bound to the peptide (which remains at the origin). Percentage of unbound ^{99m}Tc was expressed as CPM in the origin half of the strip divided by the total CPM, with all measures corrected for background.

Thin layer chromatography of the preparation of Example 13 in saline over heat-activated silica-gel coated cellulose (ITLC-SG paper) showed essentially all radioactivity associated with the peptide (R_f = 0). The preparations did not contain significant amounts of unbound ^{95m}Tc as pertechnetate or ^{99m}Tc-tartrate (R_f = 1.0).

TABLE 5. Elution of ^{99m}Tc-YIGSR-peptide preparation of Example 13 from C_{18} reverse-phase columns by increasing concentrations of ethanol. Tartrate was used in the kits as a ^{99m}Tc transfer agent. In the absence of peptide tartrate retains ^{99m}Tc, and its elution is provided here as a reference.

Percent EtOH in Eluent	PERCENTAGE OF TOTAL RADIOACTIVITY ASSAYED	
	^{99m}Tc-Tartrate	^{99m}Tc-Peptide
0 %	90.9 %	0.0 %
10 %	2.2 %	8.9 %
20 %	1.6 %	58.7 %
30 %	0.8 %	19.5 %
40 %	0.5 %	4.9 %
50 %	0.8 %	1.3 %
60 %	0.5 %	1.8 %
100 %	1.1 %	0.9 %
On Column	1.6%	4.0 %

EXAMPLE 16 - BIODISTRIBUTION OF ^{99m}Tc-YIGSR-CONTAINING PEPTIDE IN RODENTS

The biodistribution of the ^{99m}Tc-YIGSR-peptide of Example 13 was evaluated in adult female Swiss-Webster mice (approximately 19 g) at selected times (10, 30, and 120 minutes) after injection into the tail vein. Each experimental group was composed of at least five animals, with each animal receiving 0.1 ml containing 5 µg of peptide (1 µCi/µg). Animals were sacrificed by Halothane overdose, and selected organs dissected, weighed, and associated radioactivity determined. Data were analyzed using a computer program specifically

designed for ^{99m}Tc-labeled preparations. The percent dose per organ for blood, bone, and muscle were calculated assuming 7, 8.2, and 40% of total body weight, respectively, for these tissues.

TABLE 6. Biodistribution of ^{99m}Tc-YIGSR-peptide of Example 13 in normal Swiss-Webster mice at selected times after injection. All values are the mean ± standard deviation. n=6 for all data points except 120 minutes, where n=5.

	% INJECTED DOSE/ORGAN		
ORGAN	10 MINUTES	30 MINUTES	120 MINUTES
blood	7.8 ± 1.5	2.4 ± 0.3	1.5 ± 0.2
stomach	0.5 0.1	0.2 0.1	0.1 0.1
sm. intestine	4.2 1.6	7.8 0.9	1.9 0.2
appendix	0.3 0.1	0.1 0.0	2.0 0.8
lg. intestine	0.6 0.4	0.2 0.0	1.0 0.3
liver	4.9 1.3	3.4 0.4	1.3 0.0
spleen	0.1 0.0	0.0 0.0	0.0 0.0
kidneys	11.8 1.9	7.7 0.6	5.2 0.8
heart	0.3 0.13	0.1 0.0	0.0 0.0
lungs	0.6 0.1	0.2 0.0	0.1 0.0
bone	2.9 0.4	0.9 0.1	0.6 0.2
muscle	14.4 1.5	3.7 1.1	2.1 0.5
thyroid	0.1 0.0	0.0 0.0	0.0 0.0

Some studies involved pre-incubation of the ^{99m}Tc-YIGSR-peptide in whole blood prior to injection and determination of biodistribution (Table 7). In these studies, whole human blood was obtained from a healthy adult male donor and collected into Vacutainer tubes containing EDTA. After mixing to insure proper dissolution of the EDTA, approximately 2.5 ml of the whole blood was removed and mixed with 0.25 ml of ^{99m}Tc-YIGSR-peptide. The mixture was allowed to incubate 30 minutes at room temperature. After 30 minutes, aliquots of 0.1 ml were injected into the tail vein of the mice. The amount of radioactivity in the circulation for ^{99m}Tc-YIGSR-peptide of Example 13 pre-incubated in whole blood was similar to that in animals receiving ^{99m}Tc-YIGSR-peptide without incubation in blood.

TABLE 7. Biodistribution in normal Swiss-Webster mice of ^{99m}Tc-YIGSR-peptide of Example 13 after a 30 minute pre-incubation in whole blood. All values are the mean ± standard deviation. n=5 for all data points.

	% INJECTED DOSE/ORGAN	
ORGAN	10 MINUTES	30 MINUTES
blood	9.9 ± 4.7	3.7 ± 0.7
stomach	0.6 0.1	0.3 0.0
liver	5.3 1.0	4.3 1.3
spleen	0.1 0.0	0.1 0.0
kidneys	11.4 2.7	9.0 1.4
heart	0.3 0.1	0.1 0.0
lungs	0.7 0.3	0.3 0.0
bone	3.4 1.2	1.2 1.2
muscle	15.8 4.5	5.7 1.9
thyroid	0.1 0.1	0.0 0.0

The clearance rates of the ^{99m}Tc-YIGSR-peptide of Example 13 was evaluated in adult female Sprague-Dawley rats at 2 hours after injection. Each experimental group was composed of three animals. Each animal was anesthetized with ketamine and the bile duct and bladder were cannulated. Blood was collected over various periods of time from the jugular vein. The ^{99m}Tc-YIGSR-peptide cleared very rapidly from the plasma of rats, with a clearance rate of 2.6 ml/minute. At two hours, 31.2 ± 9.1% of the injected doses had cleared through urine, while bile clearance at the same time point was 6.9 ± 0.3%. In biodistribution studies, the highest amount of radioactivity was found in the kidneys, and is consistent with the rat clearance data. No major accumulation of radioactivity was found in any organ examined (other than kidney), and at later times (2 and 4 hours post injection) no re-distribution of the radiolabel was noted.

EXAMPLE 17 - IN VITRO BINDING OF ^{99m}Tc-YIGSR-PEPTIDE TO PLATELETS AND COLON CARCINOMA CELLS

The YIGSR-containing peptide of Example 13, labeled with ^{99m}Tc as in Example 14, was used to measure relative binding to colon carcinoma cells, platelets and induced clots. In these studies, ^{99m}Tc-human IgG was used as a control. Measures were expressed as a percent of final counts per minute, using the control ^{99m}Tc-human IgG as 100%.

For studies of LS-174T binding, cells were grown in cell

culture. For studies of binding to platelets and induced clots, normal whole human blood was collected in citrated buffer, and the platelet-rich plasma collected by differential centrifugation. Platelets were either used directly, or were clotted. For clot studies, 2 drops of a saturated solution of calcium chloride and magnesium chloride were added to 1 ml of platelet-rich plasma, clots were allowed to form, rinsed in buffer, and placed in phosphate buffered saline containing 1% bovine serum albumin. In all experiments, the ^{99m}Tc-YIGSR-peptide and ^{99m}Tc-human IgG preparations were allowed to incubate for 30 minutes at 37°C with the carcinoma cells, platelets and clots. For cells and platelets, separation preparatory to counting was by centrifugation; for clots, separation was by washing.

TABLE 8 Binding of ^{99m}Tc-YIGSR-containing peptide and ^{99m}tc-human IgG to LS-174T colon carcinoma cells

Sample	Final Binding (CPM)	Percent of Control
EXPERIMENT ONE (n=3)		
^{99m}Tc-Human IgG (control)	40,568±11,275	100%
^{99m}Tc-YIGSR-Peptide	98,194±30,422	242%
EXPERIMENT TWO (n=3), REPEAT OF EXPERIMENT ONE		
^{99m}Tc-Human IgG (control)	91,198±41,545	100%
^{99m}Tc-YIGSR-Peptide	276,977±21,828	304%

TABLE 9. Binding of ^{99m}Tc-YIGSR-containing peptide and ^{99m}Tc-human IgG to human platelets and clots

Sample	Final Binding (CPM)	Percent of Control
EXPERIMENT ONE (n=3), PLATELETS IN PLATELET-RICH PLASMA		
^{99m}Tc-Human IgG (control)	76,986±12,173	100%
^{99m}Tc-YIGSR-Peptide	243,269±43,838	315%
EXPERIMENT TWO (n=3), CLOTS		
^{99m}Tc-Human IgG (control)	28,331± 7,233	100%
^{99m}Tc-YIGSR-Peptide	159,763±22,314	564%

In other experiments, the peptide of Example 13, labeled as in Example 14, was used for in vitro binding studies. In these studies, the ^{99m}Tc-YIGSR-containing peptide was incubated for 90 minutes with either 0.5 ml of whole blood clots, using from 0.4 to 50 µg of radiolabeled peptide, or with approximately 10^7 platelets, using from 2 to 200 µg of radiolabeled peptide. These studies showed a dose-response relationship between the amount of radiolabeled peptide and the blood clots or platelets.

EXAMPLE 18 - IN VIVO LOCALIZATION IN INDUCED CLOTS WITH ^{99m}Tc-YIGSR CONTAINING PEPTIDE

Experimental jugular thrombi were induced in adult Fisher 344 rats using 20 µg/0.1 ml of thrombin. Studies were conducted using quantitative whole body autoradiography. The YIGSR-containing peptide of Example 13, labeled with ^{99m}Tc as in Example 14, was injected intravenously, with whole body autoradiography conducted 90 minutes after injection. Whole body autoradiography showed rapid clearance of radioactivity through or by kidneys, and to a lesser degree, through the biliary system. Significant accumulation of radioactivity was noted in the induced jugular thrombi, with a thrombus to muscle ratio of 15:1.5, and a thrombus to blood ratio of 3:1.

EXAMPLE 19 - MODIFIED YIGSR-CONTAINING PEPTIDE WITH MULTIPLE RECOGNITION UNITS

A peptide with a longer sequence to improve blood retention and repeated sequences of YIGSR to improve binding to platelets is synthesized. Synthesis is done by solid-phase synthesis techniques using t-butyloxycarbonyl (Boc) protected amino acids added sequentially to a Gly-resin ester, followed by reverse-phase HPLC purification. The sequence of the peptide is as follows:

CDGGGYIGSRGGGIGSRGGGDC

(Cys-Asp-Gly-Gly-Gly-Tyr-Ile-Gly-Ser-Arg-Gly-Gly-Tyr-Ile-Gly-Ser-Gly-Gly-Gly-Arg-Cys)

The foregoing peptide has a purity of greater than 98% as determined by reverse phase HPLC. The amino acid composition is confirmed by amino acid analysis.

The foregoing peptide is dissolved directly in nitrogen-purged 10 mM/40 mM tartrate/phthalate buffer, pH 5.5 (P/T buffer). The dissolved YIGSR-containing peptide is adjusted to a final concentration of 1 mg/ml in 10 mM P/T buffer containing 40 µg/ml of stannous tartrate and stored frozen, under a nitrogen atmosphere, in 5 cc amber serum-vials until labeled. For labeling, a vial is allowed to come to room temperature and ^{99m}Tc, as sodium pertechnetate, is added. The labeling reaction is allowed to proceed for 30 minutes. Essentially all of the ^{99m}Tc is complexed to the peptide as determined by HPLC analysis.

EXAMPLE 20 - MODIFIED YIGSR-CONTAINING PEPTIDE WITH MULTIPLE RECOGNITION UNITS AND D-AMINO ACID SEQUENCES

A peptide containing D-amino acid sequences is used to confer metabolic resistance for in vivo use. The peptide of Example 19 is modified to include such D-amino acid sequences. Synthesis is done by solid-phase synthesis techniques using t-butyloxycarbonyl (Boc) protected amino acids added sequentially to a Gly-resin ester, followed by reverse-phase HPLC purification. The sequence of the

peptide is as follows:

(D)-Cys-(D)-Asp-Gly-Gly-Gly-(D)-Tyr-Ile-Gly-(D)-Ser-Arg-Gly-Gly-(D)-Tyr-Ile-Gly-(D)-Ser-Gly-Gly-Gly-Asp-(D)-Cys

The forgoing peptide has a purity of greater than 98% as determined by reverse phase HPLC. The amino acid composition is confirmed by amino acid analysis.

The foregoing peptide is dissolved directly in nitrogen-purged 10 mM/40 mM tartrate/phthalate buffer, pH 5.5 (P/T buffer). The dissolved YIGSR-containing peptide is adjusted to a final concentration of 1 mg/ml in 10 mM P/T buffer containing 40 µg/ml of stannous tartrate and stored frozen, under a nitrogen atmosphere, in 5 cc amber serum-vials until labeled. For labeling, a vial is allowed to come to room temperature and ^{99m}Tc, as sodium pertechnetate, is added. The labeling reaction is allowed to proceed for 30 minutes. Essentially all of the ^{99m}Tc is complexed to the peptide as determined by HPLC analysis.

EXAMPLE 21 - PREPARATION OF LYOPHILIZED YIGSR-CONTAINING PEPTIDE RADIOLABELING KITS

YIGSR-containing peptide radiolabeling kits of Examples 13, 19 or 20 are prepared, with the addition of glycine and inositol as excipients. The kits are then individually vialed and lyophilized.

EXAMPLE 22 - ANIMAL LOCALIZATION STUDIES USING ^{99m}Tc-YIGSR-CONTAINING PEPTIDES

The YIGSR-containing peptide kits of Examples 13, 19, 20, or 21 are used in animal localization studies of induced pulmonary thromboembolism in adult Swiss-Webster mice with collagen/adrenaline-induced pulmonary embolism. Immediately prior to use in the studies, the animals are anesthetized by an intramuscular injection of pentobarbital. Each mouse is injected with 0.1 ml of saline containing 10 µg of collagen and 5 µg of adrenaline. This treatment results in the aggregation of circulating platelets and the subsequent lung deposition of emboli. Animals so treated exhibit 20-30% thrombocytopenia relative to control animals. Control animals receive sham injections of 0.1 ml saline. After an appropriate amount of time to allow for the development of pulmonary thromboembolism (5-15 minutes), the animals are injected with ^{99m}Tc-YIGSR-containing peptides of Examples 13, 19, 20, or 21, and 10 and 30 minute biodistribution studies are performed.

EXAMPLE 23 - DIAGNOSTIC IMAGING OF THROMBOSIS USING ^{99m}Tc-YIGSR-CONTAINING PEPTIDE KITS

A kit of Examples 13, 19, 20, or 21 is used in to localize thromboembolism in a patient. After radiolabeling with ^{99m}Tc as in Example 14, the ^{99m}Tc-YIGSR-peptide is injected intravenously. Starting immediately upon injection, the patient is imaged, using

conventional gamma scintigraphy or SPECT imaging, and is imaged at 30 minute intervals thereafter. Sites of thromboembolism will appear as photon-rich image locations by scintigraphy consistent with circulatory distribution.

EXAMPLE 24 - OTHER RADIOLABELED PEPTIDES

In addition to the specific examples above, the methods of this invention have been successfully applied to the following peptides:

a) angiotensin I,
b) renin substrate tetradecapeptide,
c) hypercalcemia of malignancy factor fragment 1-16,
d) parathyroid hormone fragment 1-34,
e) poly(histidine-glutamic acid)-poly-alanine-poly-lysine, and
f) additional chemotactic peptide analogs.

EXAMPLE 25 - ^{67}Cu-LABELED ANTIBODY-BASED PREPARATIONS

Preparation of antibodies for labeling

Human gamma globulin was obtained commercially (Gamimune^{R} N, Cutter Biological, Elkhart, IN). The method used to prepare the antibody involved reduction by a 21-hour incubation in stannous ions (2 mM). The antibody was used at a concentration of 5 mg/ml and in a buffer composed of 10 mM tartrate and 40 mM phthalate, pH 5.5, containing 2 mM stannous tartrate. In some samples, 0.5 mM $CuCl_2$ was included in the reduction buffer. The reduction step was followed by buffer exchange by chromatography through Sephadex G-25 (0.9% NaCl was used for equilibration and elution). The antibody solution was then adjusted to 1.25 mM stannous tartrate by adding an appropriate amount of 10 mM tartrate/40 mM phthalate buffer, pH 5.5, containing 5 mM stannous tartrate. This method resulted in the preparation of vials containing antibody with stannous ion-reduced disulfide bonds (thiolate groups). The antibody solution in each vial contained: a) 0.5 - 1.0 mg of antibody, and b) a buffer composed of 10 mM tartrate and 40 mM phthalate, pH 5.6, containing 1.25 mM stannous tartrate and excipients. Both the tartrate and phthalate are dicarboxylic acids used, in part, as weak chelators and stabilizers for both the stannous ions and the radiometal.

Non-reduced IgG was prepared under identical conditions, except that no stannous ions were included in the antibody reduction step. In some cases, the non-reduced IgG was incubated for 15 minutes in 0.5 mM $CuCl_2$ (approximately 10,000 molar excess). Stannous tartrate (1.25 mM) was included in the final formulation.

Antibody Labeling with ^{67}Cu

^{67}Cu [37 MBq (1 mCi)] was obtained from the University of Missouri Research Reactor (St. Louis, MO) in 0.1 M HCl. Information

on specific activity was not provided by the supplier, but was determined to be approximately 270 μCi/μg as measured by directly-coupled plasma emission spectroscopy. The volume of the ^{67}Cu solution was reduced to dryness under a stream of argon. In these experiments the relative amount of copper that was used was 2 μg of copper/mg of antibody. The dried residue was dissolved in 10 mM tartrate/40 mM phthalate buffer, pH 5.6, and the pH readjusted to 5.6 by the dropwise addition of 0.1 M NaOH.

To label kits with ^{67}Cu, lyophilized kits of human IgG were dissolved in 0.5 ml of 0.9% NaCl. To the dissolved IgG was added 1 ml of the ^{67}Cu solution as prepared above. The mixture was allowed to incubate at room temperature for up to 60 minutes. An incubation period of 15 - 20 minutes provided slightly enhanced radiolabeling yields.

IgG, which had been previously reduced by exposure to stannous ions, bound ^{67}Cu extremely well. Analysis of the recoveries from chromatography in Sephadex G-25 revealed that greater than 95% of the offered ^{67}Cu was incorporated into the antibody, which eluted in the void volume (Table 10). The high binding efficiency was confirmed by TLC in 85% methanol where it was determined that 95% of the offered ^{67}Cu was bound to the antibody. By contrast, ^{67}Cu was found to bind poorly to non-reduced IgG as determined by filtration through Sephadex G-25, a result which was confirmed by TLC (Table 10). Because less than 10% of the offered radioactivity was found to be associated with the non-reduced IgG, extended analyses were not performed with the non-reduced IgG.

TABLE 10

Comparison of percent radioactivity using native (unreduced) human gamma globulin and stannous ion reduced human gamma globulin and assayed in various in vitro tests. Thin layer chromatography (TLC) was performed using 85% methanol as a developing solution. Binding to protein A was performed using affinity columns of protein A-agarose. "ND" means not determined.

Test	Native IgG	Reduced IgG
TLC, Amount at Origin	ND	95%
Sephadex G-25 Recovery	2%	95%
Protein A-Binding	ND	89%

Aliquots of the ^{67}Cu-reduced IgG preparation (post Sephadex G-25 chromatography) were chromatographed over Protein A-agarose to estimate the amount of antibody-associated ^{67}Cu. In three separate experiments, nearly 90% of the offered radioactivity was bound to the protein A-agarose column and was not eluted by large volumes of phosphate buffered saline, pH 7.4. Elution at pH 3.0 in 0.1 M

glycine/HCl resulted in the elution of essentially all (96%) of the ^{67}Cu from the protein A-agarose columns.

In initial displacement experiments using amino acid challenge followed by TLC analysis, ^{67}Cu-IgG was challenged with graded concentrations of either cysteine or penicillamine. In these experiments, cysteine challenge (1, 2, 5, 10 and 20 mM), but not penicillamine challenge, resulted in a concentration dependant displacement of the bound copper. At concentrations greater than 10 mM cysteine more than 70% of the ^{67}Cu was displaced, while penicillamine at concentrations as high as 20 mM resulted in less than 8% displacement.

EXAMPLE 26 - ANTIBODY LABELING WITH ^{111}Ag

Human IgG (Gamimune^{R}, Cutter Biological, Elkhart, IN) was used as a source of IgG and was used without additional purification. To prepare stannous-ion reduced antibody (IgG-r), the stock solution of IgG was diluted to 8.3 mg/ml in chilled, nitrogen-purged 10 mM tartrate/40 mM phthalate buffer, pH 5.6 (P/T buffer) and the resulting solution mixed (3:2) in an amber vial with P/T buffer containing 5 mM stannous tartrate. The head-space gas was purged with nitrogen, the vial sealed, and the reaction allowed to proceed for 21 hours at room temperature. At the end of the incubation period, the solution was filtered through a 0.22 micron filter and chromatographed over Sephadex G-25 pre-equilibrated in P/T buffer, thereby removing tin ions. The protein concentration was determined colorimetrically and the IgG-r mixed (7:3) with P/T buffer containing 1.25 mM stannous tartrate and excipients. Aliquots of 0.5 ml were dispensed into individual vials and lyophilized. Upon rehydration with 0.5 ml of water each kit contained 0.5 mg of IgG-r, 40 mM phthalate, 10 mM tartrate, and 22 µg of stannous tartrate.

Human IgG lyophilized direct labeling kits, prepared as set forth above except that all sources of chloride ions are avoided, are radiolabeled by the addition of 10 mCi of ^{111}Ag.

EXAMPLE 27 - ANTIBODY BINDING SITE BLOCKING USING METALS

An anti-CEA antibody was reduced using dithiothreitol. The reduced antibody was then separated into several aliquots, and different aliquots had Hg^{+2}, Zn^{+2}, Sn^{+2} or Cu^{+2} added. The addition of the metal ions caused precipitation of the dithiothreitol, which was removed by centrifugation. The supernatant, which contained the reduced antibody and residual metal ion, was passed through a desalting column. A solution containing nitrogen purged, 40 mM sodium phthalate, 10 mM sodium tartrate, and 1.25 mM stannous tartrate was added to give 22 µgm of stannous ion per 0.5 mg of protein. 0.5 mCi of sodium pertechnetate solution was then added to

each of the different metal-treated protein preparations and binding of the radiolabeled antibody preparation to solid phase antigen (RhoChek™, RhoMed, Albuquerque, NM) was measured. The antibody preparation made with Sn^{+2} and Zn^{+2} yielded over 60% specific ^{99m}Tc binding, which is the equivalent of the result obtained with antibodies directly labeled with ^{99m}Tc by the stannous reduction method disclosed in U.S. Patent 5,102,990, entitled *Direct Radiolabeling of Antibodies and Other Proteins with Technetium or Rhenium*; however, the antibody preparations made with Cu^{+2} or Hg^{+2} yielded only about 10% specific ^{99m}Tc binding, indicating that these metal ions bind to the same binding sites as does ^{99m}Tc, and providing evidence that metals which form strong bonds with thiolate groups, such as radioisotopes of Cu and Hg, can be used to directly label reduced proteins.

EXAMPLE 28 - ^{67}Cu-LABELED ANTIBODY PREPARATIONS

Direct labeling kits were made by the methods described in Example 26. ^{67}Cu was found to be effectively bound to intact antibodies (polyclonal human IgG), as well as to antibody fragments (monoclonal anti-CEA). In these studies, greater than 98% of the ^{67}Cu was bound to the antibody, as determined by thin layer chromatography. With the polyclonal human IgG preparation, approximately 80% of the labeled material bound to protein A; with the monoclonal antibody fragment preparation, approximately 30% of the material was immunoreactive.

EXAMPLE 29 - LABELING OF HUMAN IgG WITH ^{111}In CHLORIDE

Human IgG direct labeling kits were made by the methods described in Example 26. ^{111}In chloride in 0.1 M HCl was obtained commercially. The ^{111}In containing solution was added to 10 mM tartrate/40 mM phthalate buffer, pH 5.6, and the pH readjusted to 5.6 by the dropwise addition of 0.1 M NaOH. Radiolabeling was performed by reconstituting the freeze-dried kits with 0.9% saline (U.S.P.), then adding the buffered and pH-adjusted ^{111}In. The labeling reaction was allowed to proceed for 30 minutes. At the end of the 30-minute reaction period, the mixture containing the radiolabeled antibody was analyzed by high pressure liquid chromatography. By monitoring the radioactivity and the absorbance at 280 nm, it was determined that ^{111}In co-eluted with the IgG, indicating that binding had occurred. Analysis of the overall profile indicated that 59% of the analyzed radioactivity was associated with the antibody. Thin layer chromatography on siliconized paper, using 85% aqueous methanol as a solvent, revealed that less than 3% ^{111}In was free.

EXAMPLE 30 - LABELING OF HUMAN IgG WITH CHELATED ^{111}In

Human IgG direct labeling kits were made by the methods described in Example 26. Chelated ^{111}In was purchased commercially as ^{111}In-oxine. The ^{111}In-containing solution was added to 10 mM

tartrate/40 mM phthalate buffer, pH 5.6. The lyophilized kits were radiolabeled by reconstituting the kits with 0.9% saline (U.S.P.), then adding the buffered, chelated ^{111}In. The labeling reaction was allowed to proceed for 60 minutes.

At the end of the 60-minute reaction period, the mixture containing the radiolabeled antibody was analyzed by high pressure liquid chromatography. By monitoring the radioactivity and the absorbance at 280 nm, it was determined that ^{111}In co-eluted with the IgG, indicating that transchelation had occurred. Analysis of the overall profile indicated that 65% of the analyzed radioactivity was associated with the antibody. Thin layer chromatography on siliconized paper, using 85% aqueous methanol as a solvent, revealed that approximately 3% of the ^{111}In was free.

EXAMPLE 31 - LABELING OF HUMAN IgG WITH ^{72}As

Human IgG direct labeling kits are made by the methods described in Example 26. ^{72}As is a positron emitter which can be produced from a generator system. ^{72}As is normally supplied in 0.1 M HCl. The ^{72}As-containing solution is added to 10 mM tartrate/40 mM phthalate buffer, pH 5.6, and the pH readjusted to pH 5.6 by the addition of 0.1 M NaOH. The lyophilized kits are radiolabeled by reconstitution with 0.9% saline (U.S.P.), then adding the buffered, pH-adjusted ^{72}As. The reaction is allowed to proceed for up to thirty minutes. At the end of the incubation period, the ^{72}As is bound to the IgG. Any residual unbound radionuclide is separated from the radiolabeled antibody by molecular sieve chromatography, ion exchange chromatography, affinity chromatography or other means known in the art.

EXAMPLE 32 - ^{99m}Tc LABELING OF ANTI-SSEA-1 ANTIBODY

Anti-SSEA-1 murine monoclonal IgM antibody was produced by either the MCA-480 or B37.2.1 cell lines in murine ascites by Charles River Biotechnology Services. The ascites was purified by ion exchange filter chromatography followed by column chromatography.

The purified IgM antibody was gently reduced using a solid phase disulfide reducing column. A column from a Pierce (Rockford, IL) protein reducing kit No. 77700 G was equilibrated with 10 ml of glycine buffer (10 mM glycine/NaOH buffer in 0.15 M NaCl, pH 8.3, nitrogen purged), and then activated with 10 ml of 10 mM dithithreitol in glycine buffer. The column was then washed to remove the dithiothreitol with 20 ml of the glycine buffer. 1 ml of anti-SSEA-1 in a saline buffer at pH 8.3 at a concentration of 5 mg/ml was loaded on the column. The column was then stopped, 1 ml of glycine buffer added, and the column and contents allowed to incubate for one hour at room temperature. Following incubation, the reduced

protein was eluted using 5 ml of glycine buffer in 1 ml aliquots, with the reduced protein fractions monitored by measuring absorbance at 280 nm. A nitrogen purged solution of 40 mM phthalate, 10 mM tartrate buffer. pH 5.5, with 1.25 mM stannous tartrate, was prepared, and radiolabeling kits made by adding 150 µl of the stannous tartrate radiolabeling solution to each 0.5 mg of reduced protein fraction. The kits were then vialed and immediately frozen or lyophilized.

To label the antibody, 2.5 mCi of ^{99m}Tc in 0.25 ml of saline was added and the mixture allowed to incubate at room temperature for 30 minutes. The ^{99m}Tc-labeled antibody was analyzed by quantitative, size exclusion, high performance liquid chromatography using a TSK-G3000 column and TSK-pre column. The column effluent was analyzed for gamma radioactivity and optical density measured as 280 nanometers. The ^{99m}Tc-labeled antibody was then incubated with 10 mM DTPA in phosphate buffered saline for 1 hour at 37°C to determine if the ^{99m}Tc label was vulnerable to removal by transchelation. The ^{99m}Tc radioactivity eluted simultaneously with the IgM. The elution pattern was not altered by the incubation with DTPA indicating that the ^{99m}Tc was firmly bound to the IgM protein. The ^{99m}Tc-labeled antibody was incubated with solid phase antigen: the specific binding of the ^{99m}Tc-labeled antibody to the solid phase was 59%.

EXAMPLE 33 - ALTERNATE ANTI-SSEA-1 ANTIBODY PREPARATION

Purified anti-SSEA-1 IgM antibody is reduced by 10 mM dithiothreitol (DTT) in 10 mM glycine buffered saline at pH 8.0 for 60 minutes at room temperature and 37°C. A nitrogen purged solution of 40 mM phthalate, 10 mM sodium tartrate, pH 5.5, with 7.25 mM stannous tartrate is prepared. For each 1 ml of the DTT reduced antibody solution 2 ml of the stannous tartrate solution is added and a precipitate allowed to form and incubate for 1 hour at room temperature. The precipitate is compacted by centrifugation, under nitrogen gas, for 10 minutes and the supernatant removed and chromatographed using a size exclusion chromatography column packed with Sephadex using 10 mM glycine, 10 mM inositol, 20 mM sodium phthalate, and 5 mM sodium tartrate at pH 5.6. The molecular weight fraction corresponding to 125,000 to 200,000 Daltons is collected separately and concentrated by lyophilization. The protein concentration of an aliquot of lyophilized antibody fragments is determined. Nitrogen purged, 1.25 mM stannous tartrate in 20 mM sodium phthalate and 5 mM sodium tartrate at pH 5.6 is added so that the final product contained 22 µgm of stannous ion per 0.25 mg of protein. Aliquots of 0.25 mg of protein is placed in serum vials and either frozen or lyophilized.

To label the antibody, 2.5 mCi of ^{99m}Tc in 0.25 ml of saline is

added and the mixture allowed to incubate at room temperature for 30 minutes. The ^{99m}Tc-labeled IgM antibody fragments is analyzed as in Example 32. The ^{99m}Tc radioactivity coelutes with the antibody fragment by HPLC and the specific binding of the ^{99m}Tc-labeled fragment to solid phase antigen is 60±10%.

The anti-SSEA-1 antibody recognizes an epitope present on circulating human neutrophils. For imaging of infections or inflammation by labeling neutrophils with anti-SSEA-1, the high density of antigenic sites of the circulating neutrophils provides an immediately available primary binding site. The products of Example 32 or 33 can thus be used for the detection of occult abscess and inflammation, and other conditions involving concentrations of human neutrophils.

All of the foregoing are merely illustrative, and other equivalent embodiments are possible and contemplated. The preceding examples can be repeated with similar success by substituting the generically or specifically described reactants and/or operating conditions of this invention for those used in the preceding examples.

Although the invention has been described with reference to these preferred embodiments, other embodiments can achieve the same results. Variations and modifications of the present invention will be obvious to those skilled in the art and it is intended to cover in the appended claims all such modifications and equivalents. The entire disclosures of all applications, patents, and publications cited above are hereby incorporated by reference.

CLAIMS

What is claimed is:

1. A peptide-based pharmaceutical composition suitable for administration to a patient comprising

a peptide comprising a biological-function domain and a medically useful metal ion-binding domain; and

a metal ion labeling agent.

2. The peptide-based pharmaceutical composition of claim 1 wherein said peptide comprising a biological-function domain comprises a peptide sequence selected from the group consisting of the sequence IKVAV and the sequence YIGSR.

3. The peptide-based pharmaceutical composition of claim 1 wherein said peptide comprising a biological-function domain and a medically useful metal ion-binding domain is selected from the group consisting of

$(R_1)-[Y_1]_n-(R_2)$,

$(R_1)-[Y_1-(R_2)-Y_1]_n-(R_3)$

and $(R_1)-[Y_1-(R_2)-Y_2]_n-(R_3)$

wherein,

the medically useful metal ion-binding domain is selected from one of the group consisting of $[Y_1]_n$, $[Y_1-(R_2)-Y_1]_n$ and $[Y_1-(R_2)-Y_2]_n$ in which n is a number between 1 and about 6 and Y_1 and Y_2 are amino acids comprising at least one element selected from the group consisting of sulfur, nitrogen or oxygen which is available or can be made available for binding to metal ions, and preferably selected from the group consisting of cysteine, cystine, histidine, penicillamine, deacylated methionine, lysine, arginine, aspartic acid, glutamic acid and tyrosine;

the biological-function domain comprises at least one of the group consisting of R_1, R_2 and R_3 and further comprises an amino acid sequence containing from 1 to about 20 amino acids; and

those portions of R_1, R_2 and R_3 not comprising the biological-function domain each comprise an amino acid sequence containing from 0 to about 20 amino acids.

4. The peptide-based pharmaceutical composition of claim 3 wherein said medically useful metal ion-binding domain is selected from the group consisting of

$[Cys]_n$,

$[Cys-(R_2)-Cys]_n$,

$[Cys-(R_2)-Pen]_n$,

$[His-(R_2)-Cys]_n$,

$[His-(R_2)-Pen]_n$,

$[His]_n$

and $([His-(R_2)-His]_n$

wherein,

n is a number between 1 and about 6; and

R_2 is an amino acid sequence containing from 1 to about 20 amino acids.

5. A method of performing an administrative procedure in a patient, comprising the steps of:

a) preparing a medically useful metal ion-labeled peptide comprising a biological-function domain and a metal ion-binding domain; and

b) administering an effective amount of the medically useful metal ion-labeled peptide to the patient.

6. The method of performing an administrative procedure in a patient of claim 5, preferably wherein the administrative procedure is a diagnostic procedure which diagnostic procedure further comprises imaging by metal ion detection means, preferably by at least one method selected from the group consisting of gamma scintigraphy, specific photon emission computerized tomography, positron emission tomography and magnetic resonance imaging, wherein said peptide comprising a biological-function domain comprises a peptide sequence selected from the group consisting of the sequence IKVAV and the sequence YIGSR.

7. The method of claim 5 wherein said peptide comprising a biological-function domain and a metal ion-binding domain is selected from the group consisting of

$(R_1)-[Y_1]_n-(R_2)$,

$(R_1)-[Y_1-(R_2)-Y_1]_n-(R_3)$

and $(R_1)-[Y_1-(R_2)-Y_2]_n-(R_3)$

wherein,

the medically useful metal ion-binding domain is selected from one of the group consisting of $[Y_1]_n$, $[Y_1-(R_2)-Y_1]_n$ and $[Y_1-(R_2)-Y_2]_n$ in which n is a number between 1 and about 6 and Y_1 and Y_2 are amino acids comprising at least one element selected from the group consisting of sulfur, nitrogen or oxygen which is available or can be made available for binding to metal ions, and preferably selected from the group consisting of cysteine, cystine, histidine, penicillamine, deacylated methionine, lysine, arginine, aspartic acid, glutamic acid and tyrosine;

the biological-function domain comprises at least one of the group consisting of R_1, R_2 and R_3 and further comprises an amino acid sequence containing from 1 to about 20 amino acids; and

those portions of R_1, R_2 and R_3 not comprising the biological-function domain each comprise an amino acid sequence containing from 0 to about 20 amino acids.

8. A method of labeling a protein containing monosulfides or disulfide bonds with a medically useful metal ion to obtain stable labeling, comprising the steps of:

a) incubating the protein containing monosulfides or disulfide bonds with a first reducing agent, the period of incubation being sufficient to reduce available disulfide bonds to thiolate groups while preventing excessive fragmentation of the protein;

b) substantially removing the first reducing agent from the thiolate-containing protein;

c) adding a source of Sn (II) agent to the thiolate-containing protein in a sufficient amount to form Sn (II)-containing and sulfur-containing complexes; and

d) labeling the Sn (II)-containing and sulfur-containing complexes by adding the medically useful metal ion, whereby the medically useful metal ion displaces the Sn (II) agent

and the metal ion and thiolate-containing protein form metal ion-containing and sulfur-containing complexes.

9. A method of labeling antibody against stage specific embryonic antigen-1 with a reducable medically useful metal ion to obtain stable labeling, which labeled antibody is suitable for use for detection of occult abscess and inflammation in a patient, comprising the steps of:

a) incubating the antibody against stage specific embryonic antigen-1 with a first reducing agent, the period of incubation being sufficient to reduce available disulfide bonds to thiolate groups while preventing excessive fragmentation of the antibody;

b) purifying the reduced antibody to substantially remove the first reducing agent and impurities;

c) adding a source of Sn (II) agent to the reduced antibody in a sufficient amount to form Sn (II)-containing and sulfur-containing complexes and to reduce the medically useful metal ion, the medically useful metal ion to be added in a subsequent step; and

d) labeling the purified reduced antibody by adding the medically useful metal ion, whereby the Sn (II) agent reduces the medically useful metal ion and the reduced medically useful metal ion and reduced antibody form medically useful metal ion-containing and sulfur-containing complexes.

10. The method of claims 5, 8 and 9 wherein the medically useful metal ion comprises at least one ionic element selected from the group consisting of iron, cobalt, nickel, copper, zinc, arsenic, selenium, molybdenum, technetium, ruthenium, palladium, silver, cadmium, indium, antimony, rhenium, osmium, iridium, platinum, gold, mercury, thallium, lead, bismuth, polonium and astatine.

INTERNATIONAL SEARCH REPORT

PCT/US92/11334

A. CLASSIFICATION OF SUBJECT MATTER

IPC(5) :A61K 49/02,43/00; C07K 7/00,15/28
US CL :424/1.1; 530/391.5,530/300,311;345,402
According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. :

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	CA, A, 2,016,235 (GENERAL HOSPITAL CORPORATION) 09 November 1990, See pages 7 and 8.	1,3-5,7,10
X	WO, A, 90/15818 (ANTISOMA LIMITED) 27 December 1990, See page 2.	1,3-5,7,10
X	WO, A, 89/10760 (NEW ENGLAND DEACONESS HOSPITAL CORPORATION) 16 November 1989, See pages 6,10 and 16-18.	1,3-5,7,10

[X] Further documents are listed in the continuation of Box C. [ ] See patent family annex.

* Special categories of cited documents:

"A" document defining the general state of the art which is not considered to be part of particular relevance

"E" earlier document published on or after the international filing date

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"O" document referring to an oral disclosure, use, exhibition or other means

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Date of the actual completion of the international search

17 FEBRUARY 1993

Date of mailing of the international search report

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INTERNATIONAL SEARCH REPORT

International application No.
PCT/US92/11334

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	7TH INTERNATIONAL SYMPOSIUM ON RADIOPHARMACOLOGY, Abstracts, (1991) COX ET AL., Technetium Labelled Somato statin a Potential Agent for In Vivo Tumor Localization", p.16.	1,3-5,7,10
A	GB, A, 2,225,579 (SANDOX LTD.) 06 June 1990, See the entire document.	
X Y	WO, A, 91/17173 (CYTOGEN CORPORATION) 14 November 1991, See pages 2,3,11,36-40, and 45-58.	1,3-5, 7,10 2,6
Y	Immunology (1991), volume 72, THOMPSON ET AL., "Identification of an amino acid sequence in the Iamminan A chain mediating mask cell attachment and spreading", pp. 144-149.	2,6
X Y	WO, A, 88/07382 (CENTOCOR) 06 October 1988, See pages 9-15 and examples beginning at page 38.	8,10 9
X,P Y	US, A, 5,128,119 (IMMUNOMEDICS) 07 July 1992, See the entire document.	8,10 9
A,P	US, A, 5,116,596 (HOESCHT AKTIENGESELLSCHAFT) 26 May 1992.	
Y	US, A, 4,917,878 (THAKUR) 17 April 1990, See entire document.	9